A Study of Oxidation and Inflammation using Plaque and Plasma of Vascular Disease Patients

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Abstract

Atherosclerosis is a chronic inflammatory disease leading to the formation of vascular plaques within the major arteries. Vascular plaques consist of a collection of inflammatory cells, extracellular matrix and fibrous material. Advanced plaques are characterised by the formation of necrotic zones often containing free lipid deposits high in cholesterol esters. Though considerable work has been published on the contents of these atherosclerotic plaques very little is known about how the composition varies along the length of the plaque. Nor is it known whether the pterin, 7,8-dihydroneopterin, is released by activated macrophages at levels where it can alter the plaque development through it's antioxidant activity.

Atherosclerotic plaques were removed from patients carotid and femoral arteries during endarectomy surgery and sliced into 3-5 cm sections along the length of the plaque. For each segment the concentrations of neopterin, 7,8-dihydroneopterin, α-tocopherol, TBARS, DOPA, dityrosine, the protein carbonyls α-Aminoadipic semialdehyde (AAS) and γ-glutamic semialdehyde (GGS), 7-ketocholesterol, and cholesterol was measured. Plasma samples were taken from around the sites of coronary plaques during angioplasty procedures and the concentrations of neopterin and 7,8-dihydroneopterin was measured.

The oxidant, antioxidant and inflammatory markers analysed in the atherosclerotic plaques showed many strong positive correlations between pairs of markers. The strongest was between α-tocopherol and cholesterol with significant correlations in the majority of plaques analysed. Combined plaque data analysed by dividing the segments up into three zones, pre-bifurcation, bifurcation and post-bifurcation showed that the formation of both protein and lipid oxidation markers were significantly high in the pre-bifurcation zones while alpha tocopherol was highest in the post-bifurcation zone but failed to reach significants. The pre-bifurcation zone is thought to be associated with a low shear stress level in the plaque and therefore where the oxidants localise. Neopterin levels tended also to be high in the pre-bifurcation zones but failed to reach significants. Pterin levels of as high as 2 μM were recorded suggesting in some regions 7,8-dihydroneopterin levels may reach antioxidant levels. The high variability could suggest that pterin and therefore inflammation within the plaque is a very dynamic and variable

process. Overall the data shows that each individual plaque's composition was relatively unique and variable over the length of the plaque.

The plasma samples taken from around coronary plaques showed 7,8-dihydroneopterin levels were on average elevated 3 fold compared to controls with neopterin levels only elevated 2 fold. This suggests 7,8-dihydroneopterin oxidation was not greatly elevated in these patients and the inflammation was an ongoing process.

The study shows that 7,8-dihydroneopterin and neopterin production and release is a dynamic process in vascular disease patients. The level of variability though between patients prevents the importance of pterin production on the disease progression to be determined with the current data set.

Abbreviations

AAS	α-Aminoapidic semialdehyde
ABA	4-Aminobenzoic acid
ACN	Acetonitrile
AF	Amaurousis fugax
ANOVA	Analysis of variance
AO	Aorta
apoB	Apolipoprotein B_{100}
BCA	Bicinchoninic acid
BHT	Butylated hydroxytoulene
BSA	Bovine serum albumin
CA	Coronary artery
CHD	Coronary heart disease
Cl^-	Chloride ion
CS	Coronary sinus
Cu^{2+}/Cu^+	Cupric/copper ion
DNA	Deoxyribonucleic acid
DTPA	Diethylenetriaminepentaacetic acid
DOPA	3,4-Dihydroxyphenylalanine
e^-	Electron
EDTA	Ethylenediaminetetraacetic acid
ESS	Endothelial shear stress
FA	Femoral artery
Fe^{3+}/Fe^{2+}	Ferric/ferrous ion
GGS	γ-Glutamic semialdehyde
GTP	Guanosine triphosphate
H^+	Hydrogen ion
HCl	Hypochlorous acid
HIV	Human immunodeficiency virus
H_2O_2	Hydrogen peroxide
H_2O	Water

HPLC	High performance liquid chromatography
I_2	Iodine
ICAM-1	Intracellular adhesion molecule-1
7-KC	7-Ketocholesterol
KBr	Potassium bromide
KI	Potassium iodide
KOH	Potassium hydroxide
$L^{\bullet}$	Lipid carbon centred radical
LDL	Low density lipoprotein
LH	Lipid
$LO^{\bullet}$	Lipid alkoxyl radical
$LOO^{\bullet}$	Lipid peroxyl radical
LOOH	Lipid hydroperoxide
MCO	Metal-catalyzed oxidized
MCP-1	Monocytes chemoattratant protein-1
M-CSF	Macrophage colony stimulating factor
MDA	Malonaldehyde
MES	2-(N-Morpholino)ethanesulfonic acid
MMP	Matrix Metalloproteinases
MPO	Myeloperoxidase
$NaCNBH_3$	Sodium cyanoborohydride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaH_2PO_4	Sodium dihydrogen orthophosphate
$NaHCO_3$	Sodium hydrogen carbonate
NF-κβ	Nuclear factor-κβ
7,8-NP	7,8-Dihydroneopterin
NRP	Non radical product
O_2	Oxygen
$O_2^{\bullet-}$	Superoxide
OH^-	Hydroxyl ion
$^{\bullet}OH$	Hydroxyl radical
$^{\bullet}OOH$	Peroxyl radical
oxLDL	Oxidised low density lipoprotein

PB-DOPA	Protein bound 3,4-Dihydroxyphenylalanine
PUFA	Polyunsaturated fatty acid
$R^{\bullet}$	Radical
RH	Radical in unreactive state
ROS	Reactive oxygen species
SC-A	Scavenger receptor-A
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SMC	Smooth muscle cell
STEMI	ST-elevation myocardial infarction
TBA	2-Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TFA	Trifluroacetic acid
TIA	Transient ischemic attack
TMP	α-Tocopherol mediated peroxidation
α-$Toc^{\bullet}$	α-Tocopheroxyl radical
α-TocH	α-Tocopherol
UV	Ultraviolet
VCAM-1	Vascular cell adhesion molecule-1

Introduction

1.1 Atherosclerosis

Atherosclerosis is a progressive inflammatory disease and the leading cause of death in the developed world. Atherosclerosis is characterised by the accumulation of lipids within the walls of medium to large sized arteries, leading to the formation of atherosclerotic plaques. Advanced atherosclerotic plaques often have narrowing of the arterial lumen, which restricts and eventually blocks blood flow through the artery resulting in clinical complications. More severe clinical events such as myocardial infraction and stroke are often preceded by plaque rupture, an event where the plaque contents spills into the blood flow forming a blood clot (thrombus). This causes a sudden stoppage in the blood flow through the artery. In New Zealand cardiovascular disease accounted for 40% of all deaths in 2000 (Hay, 2004). It is also estimated to account for 11% of non fatal disease burden and 24% of disability (Ministry of Health, 2001). The main risk factors leading to advanced atherosclerotic plaque formation are elevated plasma cholesterol levels, hypertension, diabetes and smoking (Chatzizisis et al., 2007; Falk, 2006; Scott, 2004). Atherosclerosis is a slow and complex disease which progresses over decades, often without symptoms until later in life. This disease, although widely studied, is still poorly understood with debate on the actual mechanism for plaque formation and development. Understanding the processes involved in plaque initiation, progression and the resulting clinical events could greatly help toward controlling and treating this disease.

1.1.1 Plaque Development and Inflammation

Arteries consist of three layers, the intima, the media and the adventitia (Figure 1.1). Atherosclerotic plaques form within the intimal layer of the artery in response to a build up of lipoprotein particles (Lusis, 2000). The intimal layer is lined with endothelial cells that allow the low density lipoproteins (LDL) to migrate through and gather within the intima. Endothelial cells act to ensure the bodies homeostasis, but they also reside in a highly vulnerable position. Changes in the blood flow can gradually induce endothelial cell dysfunction which can increase their permeability, as well as the surface expression of cell adhesion molecules and chemokines (Poli et al., 2009). An early step in the

formation of plaques is the recruitment and adhesion of inflammatory cells to the artery endothelium. Inflammation plays a key role in the development of atherosclerosis with local inflammation initiating in the formation of plaques (Lind, 2003). This causes the migration and presence of inflammatory cells into the intima, such as monocytes and T cells (Chatzizisis et al., 2007). Monocytes are able to enter the intimal space by adhesion to the vascular cell adhesion molecule-1 (VCAM-1).

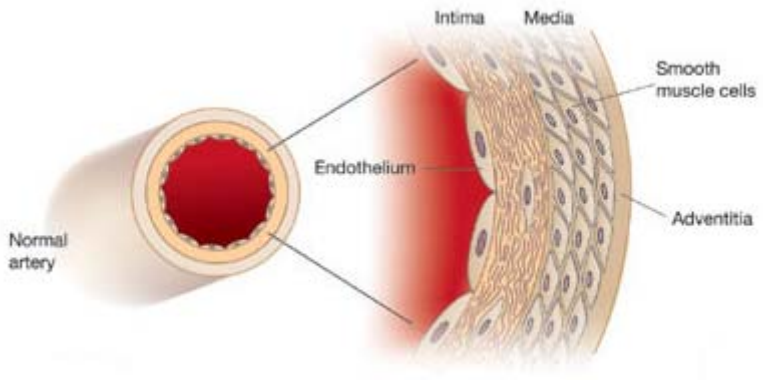

Figure 1.1 Defined Layers of the Arterial Vessel Wall
A single layer of endothelial cells provides the barrier between the arterial tissue and the flowing blood. The intima, site of plaque formation, and the media both consist of layers of smooth muscle cells with the adventitia forming the outer layer of the artery. Adapted from Libby 2002.

VCAM-1 is not expressed on healthy endothelial cells, but its up-regulation is induced by the accumulation of LDL particles and mediated by nuclear factor-κβ (NF-κβ) and pro-inflammatory cytokines which initiate the inflammatory response. (Hansson & Libby, 2006; Libby, 2002; Ross, 1999). Once bound to VCAM-1 monocytes require a chemoattractant gradient provided by the monocytes chemoattractant protein-1 (MCP-1) to migrate through the endothelial cells (Libby, 2004). MCP-1 is an inflammatory cytokine that appears to be one of the key biochemical mediators in chronic inflammatory reactions that underlie and promote the progression of atherosclerosis (Poli et al., 2009). Within the intimal space the monocytes differentiate into macrophages under the influence of macrophage colony-stimulating factor (M-CSF) (Hansson & Libby, 2006). LDL in the intima is oxidised by reactive oxygen species (ROS) to form oxidised LDL (oxLDL) through mechanisms which are poorly understood (Falk, 2006), but during the oxidation there is a modification to the apolipoprotein B-100 which renders the oxLDL susceptible to macrophage uptake (Stocker & Keaney, 2004). The

oxLDL is taken into the macrophage in a rapid and unregulated manner through the scavenger receptors CD36 and scavenger receptor-A (SC-A), leading to lipid laden macrophages known as foam cells. OxLDL is toxic to cells, and causes the death of the foam cells via necrosis. Over time these dead foam cells accumulate to form the lipid rich necrotic core.

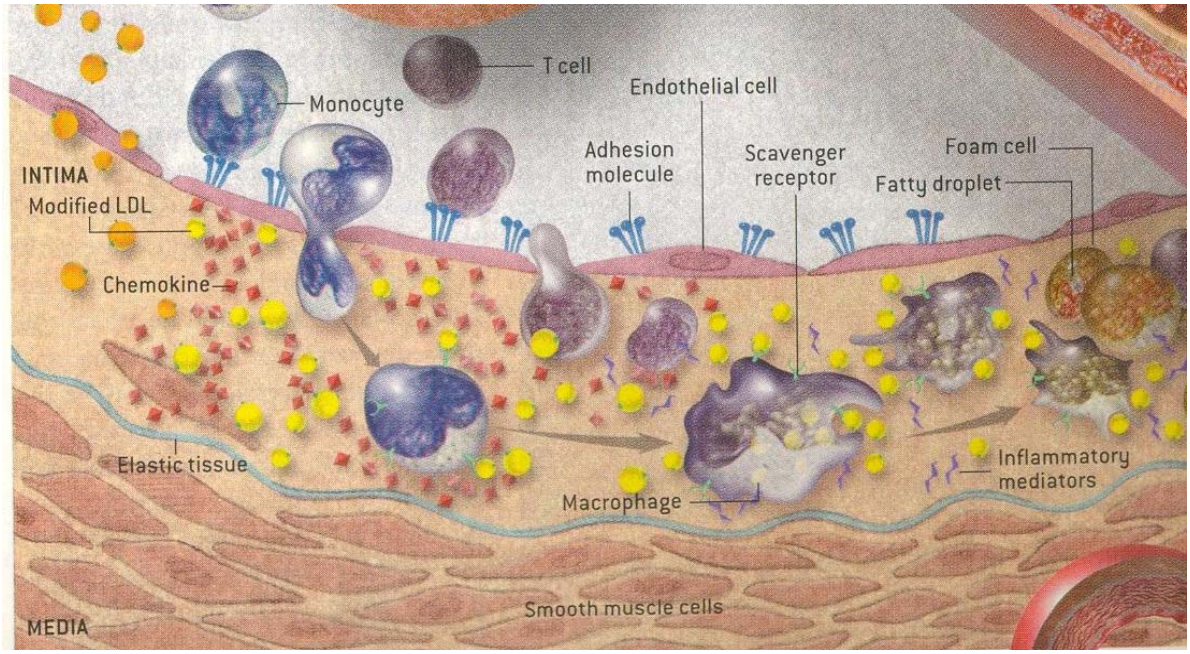

Figure 1.2 Process Involved in the Formation and Progression of Atherosclerotic Plaques
LDL and monocytes from the blood channel migrate into the intimal layer of the artery. The LDL becomes oxidised and the monocytes differentiate into macrophages. The macrophages accumulate cholesterol by the uncontrollable uptake of oxLDL leading to the formation of foam cells. Continuation of this process leads to the formation of the complex plaque. Symptoms can occur through occlusion of blood flow and/or plaque rupture. Adapted from Libby 2002b.

During this process of LDL oxidation and plaque development many biochemical markers such as oxidised lipids and proteins, anitoxidants and inflammatory markers are formed and become incorporated into the plaque. ROS within the plaque oxidises the amino acids on the apoB protein of LDL to form the protein carbonyls α-Aminoadipic semialdehyde (AAS) and γ-glutamic semialdehyde (GGS), and the protein oxidation products DOPA and dityrosine. The ROS are also able to oxidise the PUFAs and cholesterol within the LDL in macrophage foam cells to produce TBARS and 7-ketocholesterol. Inflammation occurring within the plaque activates macrophages

allowing them to produce 7,8-dihydroneopterin, which can be further oxidised to form neopterin. The concentration of these markers can be quantified along the length of advanced atherosclerotic plaques to help understand where in the plaque the majority of the oxidation and inflammation may be occurring.

1.1.2 Plaque Progression, From Early to Advanced Lesions

Atherosclerotic plaques are categorised into six levels of severity, ranging from the very early stage of lesion formation, type I, through to the advanced clinically complicated lesions, type VI. The lesions increase in size and complexity as the lipoproteins accumulate (Figure 1.3). Type I lesions (initial lesions) are characterised by the adaptive thickening of the intima and the first lipid deposits. The lipids are contained within small isolated groups of macrophages that form the first foam cells. Initial lesions are often found in children and the majority are not visible by the unaided eye (Stary et al., 1994).

Type II lesions (fatty streaks) may be seen as yellow coloured streaks on the intimal surface of an artery, although they are not always clearly visible. Intimal smooth muscle cells can contain lipid droplets but the majority of the lipids are found within macrophage foam cells which begin to form into adjacent layers (Stary et al., 1994). Type II lesions can be sub-grouped into type IIa and type IIb. Type IIa lesions are referred to as ‘advance lesion-prone’, they are found in locations with mechanical forces, such as low shear stress, often at branch points and major curvature in arteries (Stary et al., 1994; Gimborne, 1999). Low endothelial shear stress (ESS) increases the susceptibility of a lesion to progress further. Type IIb lesions are known as ‘advanced lesion-resistant’, they are found in areas where the intima is thin and contains few smooth muscle cells. Type IIb lesions either do not progress, progress slowly or progress only in people with very high plasma cholesterol levels (Stary et al., 1994).

Type III lesions (intermediate lesions) show the initiation of the necrotic lipid core. As the macrophage foam cells undergo necrosis the lipids begin to form small extracellular pools within the intima (Stary et al., 1994; Stocker & Keaney, 2004).

In type IV lesions (atheromas) the extracellular lipids form into a dense core in an extensive but well defined region of the intima. Some smooth muscles may become calcified and there is generally minimal narrowing of the lumen (Stary et al., 1995; Stocker & Keaney, 2004).

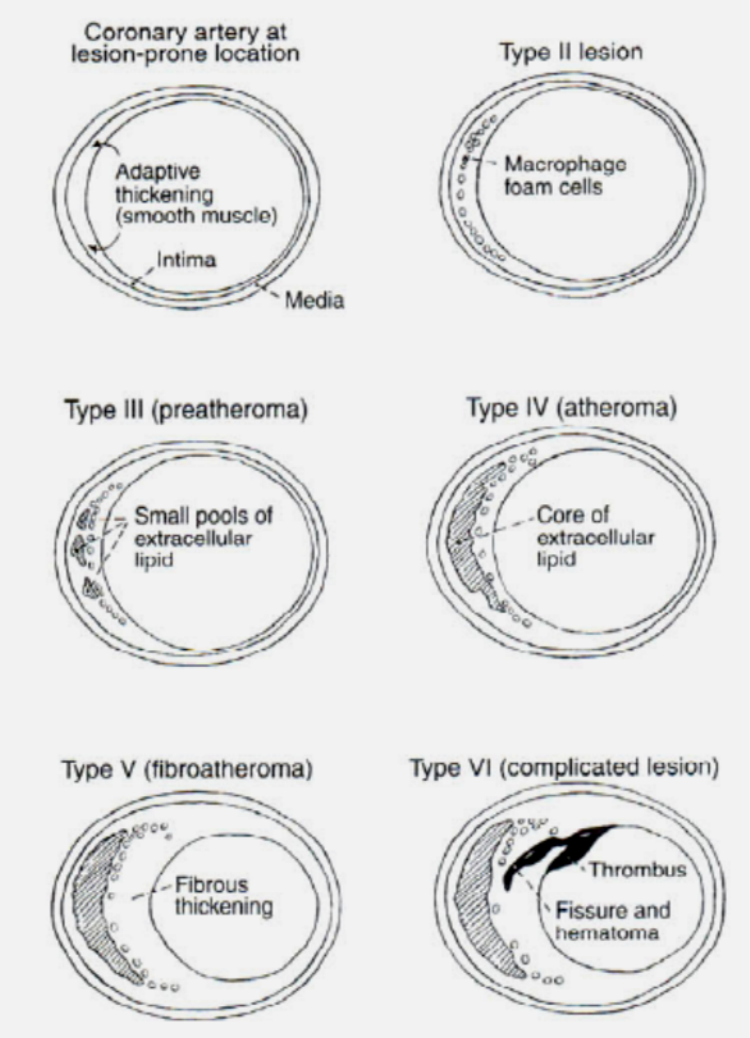

Figure 1.3 Varying Stages of Atherosclerosis
The progression of atherosclerosis is depicted from the earliest stages (top left) to the most advanced (bottom right) culminating in plaque rupture and associated thrombosis. Adapted from Stary 2004.

Type V lesions (fibroatheroma) have thick layers of fibrous connective tissue forming between the lipid core and the endothelial cells, and is what is known as the fibrous cap (Stary et al., 1995; Stocker & Keaney, 2004). As the lesion grows one of two things happen, the plaque can either undergo constrictive remodelling causing the lumen to become narrower affecting the blood flow through the artery, or the plaque could undergo expansive remodelling which causes the diameter of the artery to enlarge leaving the luminal space relatively unaffected. Expansive remodelling often results in an unstable plaque as the larger luminal circumference creates greater stress on the fibrous cap therefore increasing the likelihood that the cap will rupture (Shan, 2003; Libby, 2004; Falk, 2006).

Type VI (complicated lesions) are defined by disruptions to the lesion surface in the form of thrombus formation, haemorrhage or hematoma, all of which can cause the plaque to become even more prone to rupture (Stary et al., 1995; Stocker & Keaney, 2004).

The fibrous cap provides protection and stability to the plaque by encasing the lipid core and separating its pro-coagulant factors from the blood flow (Shan, 2003; Libby, 2004). As the plaques lipid core grows the cap begins to thin, especially at the edges, known as shoulder regions. The cap can also be broken down by matrix metalloproteinases (MMP) expressed in the plaque by macrophage foam cells that degrade to caps collagen (Shan, 2003; Jang et al., 1993). Thin, weakened caps are prone to rupture, an event that allows the lipid core to come in contact with the blood flow resulting in thrombus formation. A thrombotic event causing the blockage of blood flow through the artery is responsible for the majority of clinical events associated with atherosclerosis (Stary et al., 1995). A blockage in a coronary artery can cause a myocardial infarction or angina, a blockage in a carotid artery results in stoke or transient ischemic attack, and when the femoral arteries become blocked tissue death and gangrene can occur in the legs.

1.1.3 Endothelial Shear Stress (ESS) and Localisation of Plaques

Atherosclerotic plaques commonly form in specific areas in the arteries, such as branch points and areas of major curvature (Gimborne, 1999). This is due to these regions being under local hemodynamic forces, including blood flow-generated endothelial shear stress (ESS) (Chatzizisis et al., 2007; Gotlieb, 2005). ESS is generated thought the friction of the flowing blood on the surface of endothelial cells in the arterial wall (Chatzizisis et al., 2007). As the blood flows into the artery bifurcations the flow is disturbed, resulting in low and oscillatory ESS (Figure 1.4) (Chatzizisis et al., 2007; Ding et al., 2001). Low ESS occurs in regions of unidirectional flow and is often in the inner areas of curvatures and up stream of stenosis (Chatzizisis et al., 2007; Papaioannou et al., 2006). Oscillatory ESS causes bidirectional stress on the artery, this generally occurs at branch points and downstream of bifurcations (Chatzizisis et al., 2007).

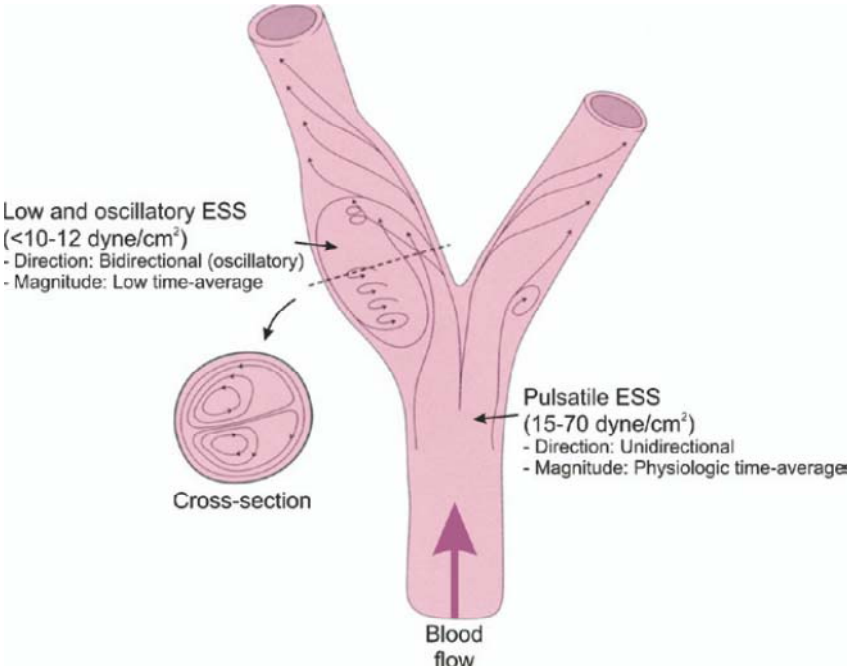

Figure 1.4 Example of Low and Oscillatory Shear Stress.
This shows the disruption to the blood at a branch point and bifurcation area resulting in endothelial shear stress. Adapted from Chatzizisis et al., 2007.

Normal ESS promotes endothelial cell stability, it has a positive affect on the cells survival and it can limit the expression of VCAM-1 by inhibiting NF-κβ activation (Libby, 2002). On the other hand, low ESS can promote endothelial dysfunction, increase monocyte binding and expression of adhesion molecules through inducing NF-κβ activation. Low ESS can also promote vascular remodelling and increase the permeability of the endothelial surface to LDL (Chatzizisis et al., 2007; Honda et al., 2001; Papaioannou et al., 2006). The low ESS in bifurcation regions allows a prolonged resident time of LDL and leucocytes on the arterial wall surface, therefore favouring their attachment and infiltration into the intima (Gimborne, 1999). Both low and oscillatory ESS have been identified to contribute to the initiation and progression of atherogenesis (Chatzizisis et al., 2007; Gimborne, 1999).

1.2 Free Radicals

Free radicals are very reactive molecules that contain an unpaired electron. They are produced continuously in cells, generally as by products of cellular metabolism (Dalle-Donne et al., 2003; de Zwart et al., 1998; Gebicki and Gebicki, 1999). The most important reactions of free radicals in aerobic cells involve molecular oxygen and its

radical derivatives to form reactive oxygen species (ROS) (de Zwart et al., 1998). Small amounts of ROS are a cellular requirement as they are involved in cell signalling pathways and defence mechanisms. Although they do not inevitably result in biological damage, an over production of ROS is potentially harmful (Dalle-Donne et al, 2003; Gebicki and Gebicki, 1999). Since ROS has the potential to induce significant biological damage, cells have many antioxidant systems for scavenging and eliminating them. Oxidative stress and cellular damage only occur when the normal homeostasis of the cell is upset and the rate of formation of ROS exceeds the capacity of the antioxidant defence system (Dalle-Donne et al., 2003; Cipollone et al., 2007). ROS can damage all types of biomolecules such as, nucleic acids, lipids and proteins. This can lead to cell death and tissue injury (Dalle-Donne et al., 2003; de Zwart et al., 1998; Levine, 2002). These highly reactive chemical species are not only important in the aging process, but they are also involved in various clinical disorders including atherosclerosis (de Zwart et al., 1998). The direct reaction of a short lived ROS with a biomolecule usually results in its oxidation and this product could be a new reactive molecule capable of oxidising or altering other biomolecules (Gebicki and Gebicki, 1999). ROS and/or their modified target biomolecules (i.e. oxLDL) can serve as second messengers and transmit extracellular signals to elevate the expression of atherogenic gene products, such as adhesion molecules and other vascular inflammatory gene products. The induced expression of these gene products promotes the infiltration of monocytes into the vessel wall and the release of additional pro-inflammatory signals which can effect inflammatory cells adhesion, migration, proliferation and differentiation (Cipollone et al., 2007; Patel et al., 2000). Both the primary and secondary products of radical damage can be used as biomarkers to monitor various disease states. Oxidised lipids and proteins are easily detected and quantified to access the level of oxidative damage occurring in biological samples.

1.2.1 Oxidation of Low Density Lipoprotein

LDL is the main carrier of free and esterified cholesterol in the blood. It is made up of 42% cholesterol esters, 22% phospholipids, 22% from the one apoB protein, 10% free cholesterol and 6% triglycerides (Esterbauer et al.1992). It is well established that one of the risk factors for atherosclerosis is high plasma LDL (Steinberg et al 1989), as LDL is oxidised and retained within the artery wall. This oxidation occurs within the arterial wall

most likely because this is an environment where the antioxidants can become depleted and the lipoproteins are exposed to oxidative stress (Berliner & Heinecke, 1996). Once oxidised the LDL contributes to atherogenesis by recruiting monocytes to the artery wall, activating the cell adhesion molecule VCAM-1 and stimulating the release of the pro-inflammatory cytokines MCP-1 and MCSF (Berliner & Heinecke, 1996; Steinberg, 1997). The oxidation of LDL produces the oxidation markers we have quantified in this study. The apoB protein on LDL contains 4536 amino acids (Steinberg, 1997), from which the oxidation of tyrosine can form DOPA and dityrosine and the oxidation of lysine, arginine and proline and form the protein carbonyl products α-Aminoadipic semialdehyde (AAS) and γ-glutamic semialdehyde (GGS). The PUFAs within the LDL are subject to free radical oxidation generating TBARS, while the cholesterol in the LDL, both free and esterified, is converted to 7-ketocholesterol via direct oxidative attack (Steinberg, 1997).

1.2.2 Lipid Oxidation

Lipid oxidation is a process occurring when the double bond in polyunsaturated fatty acids (PUFAs) is attacked by free radicals. This initiation reaction proceeds when a radical ($R^{\bullet}$) removes a hydrogen atom from the PUFA (LH), generating a fatty acid radical ($L^{\bullet}$) (Reaction 1). The fatty acid radical can then rapidly react with oxygen (O_2) to form a fatty acid peroxyl radical ($LOO^{\bullet}$) (Reaction 2). Adjacent unsaturated fatty acids (L'H) will be attracted by $LOO^{\bullet}$ promoting further oxidative damage to lipids. Oxidation is therefore a self-propagating chain reaction that can lead to the formation of lipid hydroperoxides (LOOH) (Reaction 3). The termination of this reaction occurs when fatty acid peroxyl radicals ($LOO^{\bullet}$) react with each other or endogensis antioxidants to produce non-radical products (NRP) (Reaction 4) (Cheeseman and Slater, 1993).

(1) $LH + R^{\bullet} \rightarrow L^{\bullet} + RH$

(2) $L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$

(3) $LOO^{\bullet} + L'H \rightarrow LOOH + L^{\bullet}$

(4) $LOO^{\bullet} + LOO^{\bullet} \rightarrow NR$

Lipid oxidation occurs in three distinct steps. A lag phase with little or no oxidation where the initiating or propagating radicals are removed by antioxidants, a propagation phase of increasing lipid oxidation and a final phase with a decrease in lipid oxidation, caused by more products being degraded than formed (Esterbauer et al; 1992; Halliwell and Gutteridge, 1999). Accumulation of oxidised lipid has been found in human atherosclerotic lesions from the earliest to the most advanced stages of lesion development, providing evidence of ongoing lipid oxidation within atherosclerotic lesions (Berliner, 2002; Jachec et al., 2003; Smith et al., 1992). The most widely used index of lipid peroxidation is by MDA formation, measured by the TBARS assay.

1.2.3 7-Ketocholesterol

Oxysterols, in particular 7-ketocholesterol (7-KC), are also a good marker of lipid oxidation (de Zwart et al., 1998; Fu et al., 1998). This is because during the course of LDL oxidation around 50% of its cholesterol is converted into oxysterols (Brown & Jessup, 2009). 7-KC is formed by the direct radical attack on cholesterol by ROS at the C-7 position (Brown & Jessup, 2009; Lyons and Brown, 1999). 7-Ketocholesterol is the second most abundant oxysterol found in human atherosclerotic plaques, after 27-hydroxycholesterol, and 80-95% of it is found as esters (Brown & Jessup, 2009; Lyons and Brown, 1999). Esterification of excess cholesterol in cells is mediated by acyl-CoA cholesterol acyl transferase (ACAT), this is a normal cellular mechanism for limiting the levels of free (unesterified) cholesterol in cell membranes in order to maintain normal membrane structure, ACAT also esterifies many oxidised forms of cholesterol (Brown & Jessup, 2009). It has also been suggested that some of the 7-KC present in atherosclerotic tissue and circulating plasma may come from dietary sources, as 7-KC is the major oxysterol found in cholesterol-rich foods such as meat, eggs and dairy products (Brown & Jessup, 2009; Lyons and Brown, 1999).

1.2.4 Protein Oxidation.

Proteins are major targets for free radical attack due to their abundance in cells (Davies et al., 1999; Gebicki, 1997). Free radicals are able to alter the protein structure by modification to the amino acid side chains, cleavage of the peptide backbone and DNA cross-linking (Akagawa et al., 2006; Gebicki and Gebicki, 1999). The oxidation of

proteins by free radicals and ROS can generate a range of stable and reactive products. The oxidation of aromatic side chains on tyrosine, can form DOPA and dityrosine. While a direct oxidative attack on lysine, arginine and proline can lead to the formation of protein carbonyl derivatives (Akagawa et al., 2006; Davies et al., 1999; Dean et al., 1997; Fu et al., 1998; Simpson et al., 1992). A large body of evidence suggests that protein oxidation plays a major role in a number of human diseases and conditions such as aging, Alzheimer's disease, diabetes and atherosclerosis (Akagawa et al., 2006; Dean et al., 1997, Fu et al., 1998).

1.2.5 Protein carbonyls

ROS mediated oxidative modification to amino acid side chains can produce carbonyl derivatives. Protein carbonyls are relatively stable and unreactive making them a useful marker of oxidation, they can be used to measure and quantify the oxidative damage to proteins in biological samples (Dalle-Donne et al., 2003; Davies et al., 1999). It has been confirmed that carbonyl derivatives can accumulate on tissue proteins during ageing, with the carbonyl content increasing dramatically after the sixth decade of life reaching a level where on average one out of every three protein molecules carries a modification (Akagawa et al., 2006; Levine, 2002). This increase in protein carbonyl content in tissues is associated with a number of pathological disorders including rheumatoid arthritis, Alzheimer's disease, respiratory distress syndrome, Parkinson's disease and atherosclerosis (de Zwart et al., 1998). α-Aminoadipic semialdehyde (AAS) and γ-glutamic semialdehyde (GGS) are considered to be the main carbonyl products produced from metal-catalyzed oxidized (MCO) systems and are used as specific biomarkers of oxidative damage. AAS derives from the oxidation of lysine residues, while GGS originates from the oxidation of arginine and proline (Akagawa et al., 2006; Armenteros et al., 2009: Miyata et al., 1998). Both AAS and GGS can be generated by oxidative stress *in vivo*, suggesting that their measurements can provide useful information on the contribution of oxidative stress on various diseases and ageing (Akagawa et al., 2006).

1.2.6 DOPA and Dityrosine

Protein bound 3,4-dihydroxyphenylalanine (PB-DOPA) is a major reducing species produced by hydroxyl radical attack on tyrosine residues (Dean et al., 1993). Tyrosine

can be oxidised to form mainly DOPA with some dityrosine (Davies et al., 1999; Linton et al., 2001). PB-DOPA is a relatively stable species and therefore it has the ability to diffuse to other sites, allowing it to cause damage to other proteins (Rodgers and Dean, 2000). It has the potential to initiate further oxidative reactions by binding and reducing transition metals, such as iron and copper. This can mediate further redox processes which may inflict secondary damage to other biomolecules (Fu et al., 1998; Rodgers and Dean, 2000). Because of this it has been hypothesised that DOPA is involved in the progression of plaque development (Rodgers and Dean, 2000).

Dityrosine, a very stable compound, is formed when two tyrosyl radicals react together. The long lived tyrosyl radicals can be produced from the one-electron oxidation of L-tyrosine in the presence of myeloperoxidase (de Zwart et al., 1998; Heinecke et al., 1993). Due to the stability and the fluorescence of both PB-DOPA and dityrosine, they make useful markers to measure protein oxidation in tissues (Fu et al., 1998). HPLC analysis has shown that radical-damaged proteins contain significant amounts of PB-DOPA (Gieseg et al., 1993). The levels of dityrosine in atherosclerotic regions of human aorta have been shown to be clearly increased, with the highest levels reported in early fatty streaks, However the actual ratio is only one dityrosine residue to every 3300 tyrosine residues (Levine, 2002).

1.3 Antioxidants in Biological Systems

Antioxidants are capable of removing and converting free radicals and/or oxidants into a less reactive state. Antioxidant activity can occur via enzymatic removal, for example using the enzyme superoxide dismutase to remove superoxide, or through compounds like α-tocopherol and ascorbic acid which scavenge free radicals (Halliwell and Gutteridge, 1999). Plasma contains a number of antioxidants, making blood a highly protected environment against oxidative stress. Many antioxidants have been identified within atherosclerotic plaques, these include α-tocopherol, ascorbic acid, ubiquinol-10, uric acid, glutathione peroxidise, glutathione reductase and the three isoforms of superoxide dismutase (Stocker and Keaney, 2004). Despite this protection oxidative damage still occurs within plaques.

1.3.1 Antioxidants, α-Tocopherol and Ascorbic Acid

α-Tocopherol and ascorbic acid have been suggested to limit oxidative damage and therefore lower the risk of certain diseases in humans (Villacorta et al., 2007). α-Tocopherol is a major lipid soluble chain-breaking antioxidant and the most biologically active form of vitamin E (Lui et al., 2004; Suarna et al., 2006). Located in the lipid bilayer α-tocopherol can protect both the membrane and membrane-bound enzymes from oxidative stress (Martin et al., 1996). It is also capable of protecting LDL against lipid peroxidation by directly scavenging free radicals and rapidly reacting with the lipid peroxyl radical ($LOO^{\bullet}$) to form the less reactive α-tocopherol racial ($\alpha\text{-Toc}^{\bullet}$) (Reaction 5) (Kontush et al., 1996; Thomas and Stocker, 2000). α-Tocopherol (α-TocH) can also directly react with the initiating radical to prevent $LOO^{\bullet}$ formation. The $\alpha\text{-Toc}^{\bullet}$ radical can then be eliminated by a radical-radical reaction with another $LOO^{\bullet}$ generating a non radical product (Reaction 6) (Bowry and Stocker, 1993; Thomas and Stocker, 2000). Both α-TocH and the $\alpha\text{-Toc}^{\bullet}$ have the ability to scavenge radicals, therefore each molecule of α-TocH has the potential to terminate two radicals (Bowry and Stocker, 1993; Thomas and Stocker, 2000). At high radical fluxes the concentration of $LOO^{\bullet}$ is enough for reaction 6 to predominate, resulting in both prevention of lipid peroxidation and rapid consumption of the $\alpha\text{-Toc}^{\bullet}$ radical (Bowry and Stocker, 1993). Under a low radical flux the reduced concentrations of $LOO^{\bullet}$ makes reaction 6 become infrequent and this allows α-tocopherol to switch into a pro-oxidant state (Reaction 7) (Bowry and Stocker, 1993).

α-Tocopherol is not only an antioxidant, it can also act as a pro-oxidant. One of these pro-oxidant abilities is to reduce free Cu^{2+} to Cu^{+} potentially contributing to a pool of redox active metals within the system (Yoshida et al., 1994).

(5) $\alpha\text{-TocH} + LOO^{\bullet} \rightarrow \alpha\text{-Toc}^{\bullet} + LOOH$

(6) $\alpha\text{-Toc}^{\bullet} + LOO^{\bullet} \rightarrow LOOH + \text{non radical product}$

(7) $\alpha\text{-Toc}^{\bullet} + LH \rightarrow L^{\bullet} + \alpha\text{-TocH}$

Ascorbic acid is a water soluble antioxidant which predominantly occurs in its redox active state (Halliwell and Gutteridge, 1999; Suarna et al., 1995). It is both an anti- and pro-oxidant, able to reduce metals, hydroxyl radicals and superoxide. This antioxidant is recycled via the disproportional reaction between two ascorbyl radicals (Halliwell and

Gutteridge, 1999). Ascorbic acid has been localised within plaques at concentrations higher than that detected within normal arterial tissue (Suarna et al., 1995).

1.3.2 Neopterin and 7,8-Dihydroneopterin

7,8-Dihydroneopterin (7,8-NP) and its oxidation product neopterin are produced in response to immune cell activation. This makes these both excellent inflammatory markers within the atherosclerotic plaque. When inflammation occurs in the arterial intima activated T cells release γ-interferon, this up-regulates Guanosine Triphosphate (GTP)-cyclohdrolase which converts GTP into dihydroneopterin inside activated macrophages. Intracellular phosphotates cleaves the triphosphate, this leaves 7,8-NP to diffuse out of the macrophages and into intracellular spaces and plasma (Figure 1.5) (Gieseg et al., 2008; Hamerlinck, 1999; Hoffmann et al., 2003). 7,8-NP can then be oxidised to neopterin in the presence of hypochlorite (HOCl) (Widner et al., 2000).

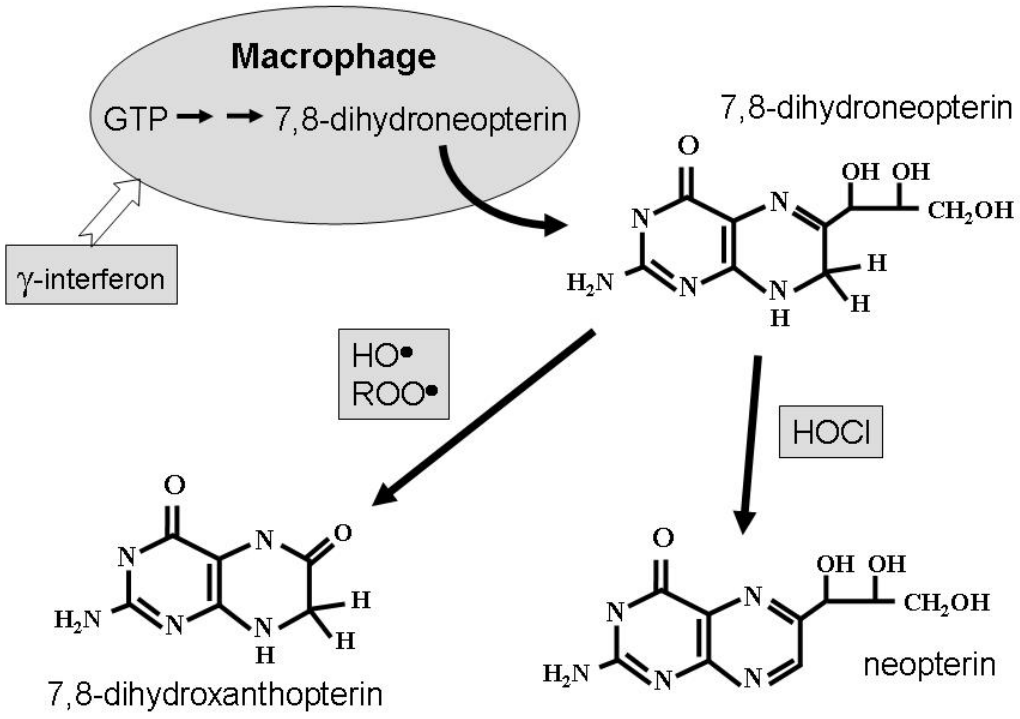

Figure 1.5 Overview of the Synthesis of 7,8-Dihyreonoepterin and Neopterin
γ-Interferon stimulation of macrophages causes the enzymatic breakdown of intracellular GTP to dihydroneopterin. This can diffuse from the cell and either be oxidised to neopterin in the presence of hypochlorous acid (HOCl) or to 7,8-dihydroxanthopterin in the presence of reactive oxygen species. Adapted from Gieseg et al. 2008.

HOCl is produced when myeloperoxidase catalyses its production from hydrogen peroxide and chloride ions in activated monocytes and neutrophiles (Reaction 8) $H_2O_2 + Cl^- + H^+ = HOCl + H_2O$ (Bergt et al., 2004; Han Kang et al., 2009; Heinecke, 1999; Widner et al., 2000). Myeloperoxidase is active in atherosclerotic lesions (Bergt et al., 2004) which suggests 7,8-NP is directly oxidised to neopterin within the plaque. Neopterin is relatively stable and highly fluorescent, making it a good marker of immune cell activation (Widner et al., 2000). Due to its fluorescence, neopterin can be easily detected in the urine and plasma by HPLC analysis (Widner et al., 2000; Flavall et al., 2008). Elevated neopterin levels occur in response to a wide range of infections, such as viral infection, bone marrow transplants, septicaemia, diabetes, cancers, HIV, arthritis, multiple sclerosis and atherosclerosis (Hamerlinck, 1999; Hoffmann et al., 2003). It has been observed that there are high correlations between neopterin increase and the severity of the inflammatory infection or malignant disease (Hoffmann et al., 2003).
7,8-NP has been reported to act as both an antioxidant radical scavenger and a pro-oxidative agent. 7,8-NP can be a potent scavenger of superoxide, peroxyl radicals (Baird et al., 2005; Duggan et al., 2002; Firth et al., 2008; Gieseg et al., 2003; Oettl et al., 1997) and nitrogen centred radicals (Oettl et al., 2000).

At micromolar concentrations 7,8-NP has been found to inhibit protein and lipid hydroperoxidation formation of LDL (Firth et al., 2008) as well as copper and peroxyl radical mediated LDL oxidation (Gieseg & Cato, 2003). The cell lines THP-1 and U937 are both protected from AAPH derived peroxyl radicals with 7,8-NP (Baird et al., 2005). 7,8-NP also protects U937 cells from Fe^{2+} and HOCl oxidation (Gieseg et al., 2003), as well as protein hydroperoxidation formation and peroxyl radical mediated thiol loss (Duggan et al., 2002). While at higher concentrations 7,8-NP acts as a pro-oxidant, it is able to induce apoptosis to several cells lines due to increased oxidative stress on the cells (Baier-Bitterlich et al., 1995; Baier-Bitterlich et al., 1997; Enzinger et al., 2002; Wirleitner et al., 2003). 7,8-NP has also been found to enhance hydrogen peroxide activity at a slightly alkaline pH (Weiss et al., 1993). The properties of 7,8-NP are therefore dependant on its concentration and chemical environment

1.4 Objective of this Study

The aim of this study is to quantify the oxidant and antioxidant content of advanced atherosclerotic plaques and examine the relationships these markers have on each other and within their localisation along the plaque. Also explore the levels of neopterin and 7,8-dihyroneopterin in patients with cardio heart disease.

Many studies have identified the concentration of antioxidants and oxidation products within atherosclerotic plaques (Brown et al., 1997; Carpenter et al., 1993; Fu et al., 1998; Iuliano et al., 2003; Jachec et al., 2003; Micheletta et al., 2004; Nishi et al., 2002; Upston et al., 2002), but none have investigated these markers in respect to their spatial localisation along the length of the plaque or within respect to each other. Our laboratory has so far been the first to quantify the amount of neopterin in femoral and carotid plaques (Firth et al 2008; Gieseg et al., 2008). Within our laboratory fourteen plaques have been fully analysed, however due to this small sample size, the complexity and the differences in the plaques advanced diseased states, it has been difficult to obtain significant trends and correlations between the plaques. I have therefore analysed a further five plaques to hopefully begin to understand the potential relationships between the markers and where along the plaques certain markers may localise.

Materials and Methods

2.1 Materials

2.1.1 Chemicals

All solutions were prepared using de-ionised and ultrafiltrated water from a NANOpure filtration system supplied by Barnstead/Thermolyne (IA, USA). All reagents used are of analytical grade or better, unless otherwise stated.

Acetic acid (glacial)	JT Baker, Mallinckrodt Baker Inc.
Acetone	Merck
Acetonitrile	JT Baker
Argon gas	BOC gasses, N.Z.
4-Aminobenzoic acid (ABA)	Sigma Chemical Co.
Ammonium Phosphate dibasic minimum 98%	Sigma Chemical Co.
Bicinchoninic acid (BCA) protein determination kit	Pierce, U.S.A
Bovine serum albumin (BSA)	Sigma Chemical Co.
Butylated hydroxytoulene (BHT)	Sigma Chemical Co.
Cholesterol reagent	Roche Diagnostics, USA
5-Cholesten-3B-ol-7-one	Sigma-Aldrich
Diethyl ether	Merck
Diethylenetriaminepentaacetic acid (DTPA)	Sigma Chemical Co.
7,8-Dihydroneopterin (7,8-NP)	Schiricks Laboratory
Ethanol	BDH Chemical Ltd
Ethylenediaminetetraacetic acid (EDTA)	BDH Chemical Ltd
n-Hexane	Unichrom, ARS
Hydrochloric acid (HCl)	Merck
Iodine	BDH Chemical Ltd
L-3,4-Dihydroxyphenylalanine	Sigma Chemical Co.

L-Ascorbic acid	Sigma Chemical Co.
Mercaptoacetic acid	Sigma Chemical Co.
Methanol	Merck
2-(N-Morpholino)ethanesulfonic acid (MES)	Sigma Chemical Co.
Neopterin	Schiricks Laboratory
Nitrogen gas	BOC gasses, N.Z.
Nitrogen liquid	Cryogenics, Department of Chemistry, University of Canterbury.
Orthophosphoric acid (85%)	BDH Chemical Ltd
Phenol	Sigma-Aldrich
Potassium hydroxide	Merck
Potassium iodide	Merck
2-Propanol (isopropanol)	Merck
Sodium acetate	Merck
Sodium cyanoborohydride ($NaCNBH_3$)	Fluka
Sodium dihydrogen phosphate monohydrate	Scharlau Chemie S.A.
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich
Sodium hydroxide	Merck
1,1,3,3-Tetramethoxypropane	Sigma Chemical Co.
2-Thiobarbituric acid, minimum 98% (TBA)	Sigma Chemical Co.
α-Tocopherol	Sigma Chemical Co.
Trichloracetic acid (TCA)	Merck
Trifluroacetic acid (TFA) anahydrous	Sigma Chemical Co.

2.1.2 HPLC Systems

Two separate HPLC units were used during the research. HPLC system 1 (Shimadzu Corporation, Japan) consists of the controller SCL-10Avp, fluorescence detector RF-10A$_{XL}$, UV-Vis detector SPD-10A, autosampler SIL-10A, as well as column oven and degasser. HPLC system 2 (Shimadzu Corporation, Japan) consists of the controller CBM-20A, fluorescence detector RF-10A$_{XL}$, diode array detector SPD-M20A, autosampler SIL-20AC$_{HT}$, plus column oven and degasser. Peak areas were determined using LC solution software.

2.1.3 HPLC Mobile Phase

Salt based HPLC mobile phases were filtered through a 45 μm filter and all mobile phases were sonicated for 10 minutes before loading onto the HPLC.

2.2 Methods

2.2.1 Plaque Homogenisation

Plaques were surgically removed at Christchurch Hospital by carotid or femoral endarterectomy on patients that had high-grade stenosis. It was generally possible to excise the plaque without entering the vessel lumen other than at the proximal and distal ends of the dissection. This usually preserved the luminal anatomy of the plaque and left the stenosis undisturbed within the excised specimen, leaving a “cast” of the plaque bifurcation. Once consent was given the excised plaques was placed on ice and immediately transported to the Free Radical Biochemistry Laboratory at The University of Canterbury where they were frozen at -80 and stored at -80 C.

When ready for analysis the plaques were sectioned into 3-5 mm segments down their longitudinal axis while still frozen. Each section was numbered beginning at the end prior to the bifurcation and photographed. The sections were homogenised to a fine powder under liquid nitrogen, and weighted. Next 3.5 mL of water, 35 μL of 100 mg/mL EDTA and 35 μL of 20 mg/mL BHT (in methanol) was added, then vortexed to mix. The final volume for each section was noted.

2.2.2 Plasma Collection

Blood samples were taken from patients with either chronic stable angina or suffering from an acute ST-elevation myocardial infarction while they where undergoing an angioplasty procedure at Christchurch Hospital. The first blood sample was taken from the femoral artery with more samples taken from in and around the culprit coronary artery using a low profile sampling catheter both before and after the blockage was removed. Twenty four hours following the procedure a 5ml blood sample was obtained from a forearm vein. Healthy volunteers each gave a 5 ml venous blood sample from the forearm. All samples were collected into EDTA tubes, iced immediately and shielded from the light. Whole blood was centrifuged at 1,200g at 4˚C for 10 minutes. The plasma was separated and acetonitrile added to give a 50:50 mix, this was then stored at – 80˚C for subsequent analysis.

2.2.3 Cholesterol Determination

This is a spectrophotomic assay based on a series of reactions involving the enzymes cholesterol esterase, cholesterol oxidase and peroxidise which results in the development of a red dye. The intensity of the dye is directly proportional to the concentration of total cholesterol within the sample. 10 μL of plaque homogenate was added to 1 mL of cholesterol reagent (CHOL, Roche Chemicals) and incubated in the dark for 10 minutes at room temperature. The absorbance was read at 500 nm against a cholesterol reagent blank.

2.2.4 Protein Determination

The protein content of a sample is determined using the BCA protein kit (Pierce, Illinois, USA). Bicinchonic acid (BCA) reacts with the protein to form a purple dye which is proportional to the protein concentration. Optimally, the protein concentration within the sample is required to be between 25 and 250 μg/mL therefore plaque homogenate was diluted with water by a factor of 10. 1 mL of freshly prepared working reagent in a 50:1 ratio of reagents A:B (Reagent A sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0.1 M sodium hydroxide and Reagent B 4% hydrated copper sulphate) was added to 50 μL of diluted plaque homogenate and placed on a heated block at 60ºC with gentle shaking for 30 minutes. The reaction was stopped by cooling samples on ice

before reading the absorbance at 562 nm against a water blank. Concentrations were determined from a BSA standard curve analysed by Prism (version 4.0, GraphPad Software, USA).

2.2.5 Antioxidant Detection

2.2.5.1 Pterin Assay

The Pterin assay takes advantage of neopterin's natural fluorescence allowing the detection and determination of neopterin and 7,8-dihydroneopterin (7,8-NP). To determine the 7,8-NP concentration it is oxidised to neopterin giving a total neopterin value, therefore subtracting the neopterin from the total neopterin gives the 7,8-NP concentration. Using reverse phase HPLC the accuracy of the assay is dependent on the removal of protein from the sample, as it has been shown that neopterin interacts with the proteins leading to an underestimation of its concentration (Flavall et al., 2008). Due to the labiality of 7,8-NP the pterin assay was preformed on the same day as the homogenisation of the plaque.

For Plaque Homogenate

For neopterin quantification the protein precipitation was induced by mixing 50 µL of ACN with 50 µL of plaque homogenate followed by vortexing and centrifugation at 20,800g for 10 minutes at 4ºC. For total neopterin samples 50 µL of ACN was added to 50 µL of plaque homogenate, vortexed and centrifuged as described above for 5 minutes. For the oxidation of 7,8-NP to neopterin 20 µL of acidic iodide (5.4%I_2/10.8% KI in 1 M HCl) was added, vortexed and incubated in the dark for 15 minutes. This was followed by 20 µL of freshly prepared 0.6 M ascorbic acid to neutralise excess acidic iodide, and centrifuged as before for 10 minutes.

From the neopterin and total neopterin supernatants, 10 µL was injected onto a reverse phase Synergi 4u Hydro-RP 80A, 250 x 4.6 mm column (Phenomenex) maintained at 35ºC. The mobile phase of 20 mM ammonium phosphate pH 6.0 with 5% methanol mobile phase was pumped at a flow rate of 1 mL/minute and the neopterin detected at an excitation of 353 nm and emission 438 nm.

For Plasma Samples

The frozen 50:50 plasma:ACN mix was defrosted under cold running water, followed by vortexing and centrifugation at 20,800g for 10 minutes at 4ºC. For the neopterin analysis, supernatant was taken directly from this ependorf and injected onto the HPLC as above. For the 7,8-NP samples 50 µL of the supernatant was placed into a separate ependorf with 10 µL of acidic iodide (5.4%I_2/10.8% KI in 1 M HCl), vortexed and incubated in the dark for 15 minutes. 10 µL of freshly prepared 0.6 M ascorbic acid was then added to neutralise excess acidic iodide, then centrifuged at 20,800g for 10 minutes. The supernatant was injected onto the HPLC exactly the same as in the plaque homogenate samples.

A standard neopterin stock stored at -20 ºC was prepared in 10 mM phosphoric acid and sonicated for 2 minutes. When required an aliquot was thawed and diluted to 50 nM with mobile phase. The 7,8-NP standard was prepared fresh in mobile phase and kept on ice and in the dark to minimise loss.

2.2.5.2 α-Tocopherol Assay

α-Tocopherol is a lipid soluble antioxidant due to its long hydrocarbon chain and efficient peroxyl radical scavenging properties. It has a cyclic structure which is important for its antioxidant activity and also allows its detection at 292 nm.

Within 10 mL glass culture tubes with screw tops 100 µL of plaque homogenate was diluted with 400 µL of water. To prevent any additional oxidation occurring 10 µL of 100 mg/mL EDTA and 25 µL of 20 mg/mL BHT (in methanol) was added. Protein precipitation was induced with 500 µL of ice cold ethanol and vortexed for 10 seconds. The α-tocopherol was extracted by adding 2 mL of hexane with further vortexing for 30 seconds. At this point the samples were stored a -80ºC until needed for analysis. Once thawed the samples were vortexed for 60 seconds before centrifuging at 600g for 5 minutes to maximise phase separation. 1.4 mL of the hexane layer was transferred into glass tapered 10 mL tubes and evaporated in a hot water bath under nitrogen gas. The remaining residue was dissolved in 100 µL ice cold methanol and injected onto the HPLC.

With a mobile phase of 100% methanol, 20 µL sample was injected onto a reverse phase Phenosphere-NEXT C-18, 150 x 4.6 nm, 5 µm column (Phenomenex) maintained at 35ºC, and detected at an extinction of 292 nm and emission of 353 nm. The α-tocopherol standard was prepared by diluting approximately 1 mg/mL α-tocopherol in methanol and the concentration determined spectrophotometrically using an extinction coefficient of 3086 $cm^{-1}M^{-1}$ at a wavelength of 294 nm. The α-tocopherol standard stock solution was stored at -20ºC under argon gas and when required was diluted to a 3 µM concentration in methanol.

2.2.6 Lipid Oxidation Measurement

2.2.6.1 TBARS Assay

The TBARS assay provides a general measurement of fatty acid oxidation occurring within biological systems. One of the breakdown products of lipid oxidation is malondialdehyde (MDA). The well established 2-thiobarbitutic (TBA) reaction with MDA yields a pink colour that is detected at 553 nm

Into a 1.75 mL centrifuge tube 100 µL of plaque homogenate was placed, with 50 µL of 150 mM phosphoric acid to increase the acidity and precipitate the protein, 10 µL of 20 mg/mL BHT (in methanol) was added to prevent further oxidation and the samples were stored at -80ºC. When ready for analysis 50 µL of freshly prepared 42 mM TBA was added to the thawed samples and placed on a heated block at 95 ºC with gentle shaking for 30 minutes allowing the formation of the TBA-MDA adduct. The samples were cooled on ice, 800 µL of cold methanol was then added, incubated on ice for a further 5 minutes and centrifuged at 20,800g for 10 minutes at 4 ºC. To equilibrate the sample to the mobile phase 100 µL of the supernatant was added to 100 µL of 50 mM NaH_2PO_4 pH 6.8 and injected onto the HPLC.

The HPLC mobile phase was 50 mM NaH_2PO_4 pH 6.8 with 45% methanol at a flow rate of 1 mL/minute through a reverse phase Phenosphere C-18, 4.6 x 150 mm, 5 µm column (Phenomenx) heated to 35ºC. The TBA-MDA was detected at an excitation of 525 nm and emission of 550 nm. Both blank and 1 µM TBA-MDA standards were required to determine the concentration of TBARS within the samples.

MDA is very labile, therefore the standard was freshly prepared with each sample block by incubating a known concentration of the MDA precursor 1,1,3,3-tetramethoxypropane with TBA. 1,1,3,3-tetramethoxypropane hydrolyses to MDA during the heated incubation step forming a known concentration of TBA-MDA. During preparation of the standard the first dilution step of 1,1,3,3-tetramethoxypropane was in a solution of ethanol:water at a 2:3 ratio, where further dilutions were made in water.

2.2.6.2 Combined 7-Ketocholesterol and α-Tocopherol Assay

To reduce the amount of homogenate used in the plaque analysis a modified procedure was developed to measure both 7-ketocholesterol and α-tocopherol with one injection onto the HPLC.

The 7-ketocholesterol concentration was determined for both the free 7-ketocholesterol and the total 7-ketocholesterol (free plus esterified). For both the free and total 7-ketocholesterol 100 µL plaque homogenate was diluted in 400 µL water, to prevent additional oxidation 10 µL of 20 mg/mL BHT (in methanol) and 20 µL of 100 mg/mL EDTA was added. Protein precipitation was induced by 500 µL of cold ethanol and vortexed briefly. The 7-ketocholesterol and α-tocopherol was extracted by the addition of 2 mL hexane, vortexed for 30 seconds and this was then stored at -80ºC until required for analysis. Once thawed the samples were vortexed for 60 sections and centrifuged at 600g for 5 minutes at 4ºC. 1.4 mL of the hexane layer was transferred into glass tapered 10 mL tubes and evaporated in a hot water bath under oxygen free nitrogen gas. For free 7-ketocholesterol and α-tocopherol analysis the remaining residue was dissolved in 100 µL ACN:isopropanol at a 4:5 ratio, and injected onto the HPLC. For total 7-ketocholesterol analysis the remaining residues was dissolved in 2 mL 20% KOH (in methanol) plus 2.5 mL diethyl ether, then placed under argon gas and vortexed. The samples were incubated on ice for 3 hours, with a brief vortex every 30 minutes. The reaction was then stopped by adding 2 mL 20% acetic acid and 2.5 mL hexane and vortexed for 60 seconds. 4 mL of the upper layer was transferred into glass tapered 10 mL tubes and dried down under oxygen free nitrogen gas, before re-solubilising in 100 µL of mobile phase.

20 µL of the samples was injected onto a Phenosphere-NEXT C18, 250 x 4.6 mm, 5 µm column (Phenomenex) maintained at 35ºC pumping a mobile phase of 54 isopropanol:44

acetonitrile:2 water at 1 mL/min. The elute first ran through the UV detector for the detection of 7-ketocholesterol at a wavelength of 234 nm, and continued on through to the fluorescence detector set at an excitation of 292 nm and emission of 335 nm for the detection of α-tocopherol.

The α-tocopherol standard was prepared by diluting approximately 1 mg/mL α-tocopherol in methanol and the concentration determined spectrophotometrically using an extinction coefficient of 3086 $cm^{-1}M^{-1}$ at a wavelength of 294 nm. The α-tocopherol standard stock solution was stored at -20ºC under argon gas and when required was diluted to a 3 μM concentration in methanol. The 7-ketocholesterol standard was prepared in the mobile phase and diluted to 10μM in a ACN:isopropanol solution at a 4:5 ratio.

2.2.7 Protein Oxidation Detection

2.2.7.1 DOPA and Dityrosine Assay

DOPA and Dityrosine are oxidative products derived from the tyrosine residue. HPLC analysis is able to separate the products after their release from the protein and allows the detection of both these protein oxidative products with one assay.

Into 7.5 mm glass durham tubes was placed 10 μL of 100 mg/mL EDTA, 10 μL of 20 mg/mL BHT (in methanol) and 100 μL of plaque homogenate. The protein was precipitated by the addition of 900 μL of ice cold acetone and incubated on ice for 10 minutes followed by centrifugation at 5000g at 4ºC for 15 minutes. The supernatant was removed and the pellet washed with 500 μL diethyl ether to remove lipids and centrifuged as before. After the removal of the supernatant the pellets were dried under vacuum for 1 hour and then placed into pico-Tag vials (Millipore, USA) with 1 mL of 6 M HCl with 1% ($^{w}/_{v}$) phenol and 50 μL mercaptoacetic acid in each vial. The vials were flushed with argon gas for 5 minutes and evacuated by connecting to the vacuum line of the solvent Speed Vac for 2 seconds. For the acid hydrolysis of the proteins the vials were incubated in a 110ºC oven for 16 hours. After cooling, the samples were centrifuged under vacuum in the Speed Vac for 2 hours before re-solubilising the pellet with 200 μL of 0.1% TFA. The samples were vortexed and transferred into ependorf

tubes to enable centrifugation at 20,800g for 10 minutes at 4ºC before injecting onto the HPLC.

Using a reverse phase Aqua C18, 250 x 4.6 mm, 5 µm column (Phenomenex) 10 µL of the acid hydrolysate was injected with a gradient mobile phase pumping at 1 mL/minute. The gradient began a 99% 0.1% TFA pH 2.5 with 1% ACN, with the ACN increasing to 5% by the 10^{th} minute, 10% by the 14^{th} minute and 50% by the 16^{th} minute. This concentration was maintained for 5 minutes to clean the column, after which the ACN concentration was returned and maintained at 1% until the 30^{th} minute. Because DOPA elutes first the fluorescence detector began at an excitation of 280 nm and an emission of 320 nm. At the 11^{th} minute the emission is changed to 410 nm for the detection of Dityrosine. The DOPA standard was prepared fresh and a Dityrosine standard stock is stored at -20ºC, both were made up and diluted in 0.1% TFA.

2.2.7.2 Protein Carbonyls AAS and GGS HPLC Assay

This method detects two of the main protein carbonyl products, α-aminoadipic semialdehyde (AAS) and γ-glutamic semialdehyde (GGS). AAS and GGS accounts of 23% of the total carbonyls within native BSA (Akagawa et al, 2006).

The samples were prepared in glass durham tubes with 200 µL plaque homogenate, to prevent further oxidative events 50 µL of 1 mM DTPA was added followed be 50 µL of 50% TCA to precipitate proteins. The samples were kept on ice for 10 minutes then centrifuged for 10 minutes at 500g at 4ºC. The supernatant was removed and using a sealed pasture pipette as a stirring rod the protein pellet was re-suspended in 125 µL of 0.25 M MES buffer pH 6.0 with 0.5% SDS. To the mixture 250 µL of the highly fluorescent 50 mM ABA in MES buffer and 125 µL of 100 mM $NaCNBH_3$ in MES buffer was added and the tubes were briefly vortexed before incubating in the dark at 37ºC at 80 rpm for 90 minutes, allowing the ABA to react with the AAS and GGS. After the samples were cooled on ice the protein was precipitated with 500 µL of ACN, incubated on ice for 10 minutes and centrifuged at 500g at 4ºC for 15 minutes. To ensure the protein was precipitated, the supernatant was removed and the pellet washed with 1 mL 10% TCA and centrifuged as before. The supernatant was removed once again and the pellet re-suspended in 1 mL of cold ethanol and spun as before. For the final time the supernatant was removed and the residual ethanol drained off by inverting the tubes for 5

minutes. The pellets were dried under vacuum for 1 hour before placing them into Pico-Tag vials with 1.5 ml 6 M HCl for acid hydrolysis. Air was flushed out using argon gas for 5 minutes before the vials were evacuated by connecting to the vacuum line of a solvent Speed Vac for 2 seconds. For the acid hydrolysis of the proteins the vials were incubated in a 110ºC oven for 24 hours. After cooling, the samples were centrifuged under vacuum in the Speed Vac for 2 hours before re-solubilising the pellet with 200 µL of 50 mM sodium acetate pH 5.4. The samples were vortexed and transferred into ependorf tubes to enable centrifugation at 20,800g for 10 minutes at 4ºC before injecting onto the HPLC.

Onto a reverse phase Synergi Fusion 250 x 4.6 mm, 4 µm column (Phenomenex) with a gradient mobile phase pumping at 1 mL/minute, 20 µL of each sample was injected. The mobile phase began at 95% of 50 mM sodium acetate pH 5.4 and 5% ACN and was maintained until the 20th minute. The ACN concentration increased reaching 50% by the 25th minute and this was maintained for 5 minutes to clean the column, after which the ACN concentration was returned to 5% for the remainder of the run (40 minutes). The AAS-ABA and GGS-ABA compounds were detected at an excitation of 283 nm and an emission of 350 nm. For the standard, ABA was prepared in mobile phase, sonicated for 2 minutes and injected at a concentration of 1 µM.

2.3 Statistical Analysis

Unless otherwise stated, the data shown represents the mean ± the standard error of the mean (SEM) of triplicates for each section. The SEM is represented on each bar and data point within the graphs. The statistical analysis was preformed using Prism (version 4.0 and version 5.0, Graphpad Software, USA) and Statistica (version 9.0 Statsoft, Inc, USA). Statistical significance are represented by * ($P<0.05$), **($P<0.01$) and *** ($P<0.001$).

Comparisons between the individual sections in each plaque were preformed by one-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison test to designate significant variations between the means. Statistics on the correlation data was preformed using Statistica's correlation matrices. All statistics on the human plasma data used one-way ANOVA followed by the Tukey's test.

2.4 Ethics Approval

Ethics approval for access to patient information and analysis of atherosclerotic plaques was granted from the Upper South A Regional Ethics Committee, ethics number CTY/01/04/036. Under this agreement consent from the donor was obtained and the plaques remained anonymous from the donor labelled by a laboratory based code.

Collection of blood from patients undergoing angioplasty was approved by the Upper South A Regional Ethics Committee, part of the Health Research Council, administered through the New Zealand Ministry of Health (Ethics Reference: URA/05/08/097). The period of patient enrolment was October 2007 – March 2009. All patients and controls provided written consent prior to enrolment and were fully informed of the study design and methods

Results

3.1 Atherosclerotic Plaques

All atherosclerotic plaques were received from patients undergoing carotid or femoral endarterectomy and were in an advanced stage of the disease. The plaques were cut into 3-5 mm long sections beginning in the direction of the blood flow. During homogenisation, the plaques were qualitatively assessed on their morphology and overall composition, including the degree of calcification and the presence of 'gruel', large areas of the lipid core.

Because the level of shear stress may play a role in the formation, progression and rupture of these plaques (Chatzizisis et al., 2007), each plaque section was classified into zones based on its location along the plaque. These zones represent the pre-bifurcation, bifurcation and post-bifurcation regions. The zones may be under different levels of shear stress and therefore represent potentially different oxidative environments. The zoning also allows for standardisation of each plaque for a combined plaque analysis.

Within each plaque section of the five atherosclerotic plaques a range of markers were measured. These markers included the inflammatory marker neopterin, as well as total pterin (neopterin plus 7,8-dihydroneopterin), lipid soluble antioxidant α-tocopherol, the general lipid oxidation marker TBARS, the oxysterol 7-ketocholesterol, protein oxidation markers DOPA, Dityrosine, and protein carbonyls AAS and GGS, along with protein and total cholesterol.

In the plaque profile graphs the clear bars represent the section/s from the pre-bifurcation zone, the solid filled bars from the bifurcation zone and the stripped bars represent the section/s from the post-bifurcation zone. Statistical analysis was carried out on all the marker analysis data, with an asterisk placed on the top of any bar/point to indicate a statistically significant change in the concentration of a section from the previous section. * represents ($P<0.05$), ** ($P<0.01$) and *** ($P<0.001$).

3.1.1 Plaque A (Plaque Laboratory No 17)

Plaque A was removed from the left carotid artery. It was characterised with over 90% stenosis, 38 mm in length and weighed a total mass of 1.179 grams. In overall composition, this plaque was soft with only slight calcification in section 6 (Figure 3.1.1). Plaque A was cut into 9 sections where sections 1-2 were the pre-bifurcation zone, sections 3-4 were the bifurcation and sections 5-9 were the post-bifurcation zone.

Table 3.1.1 Plaque A Patient and Clinical Information

Location	Left Carotid Artery	**Stenosis**	>90%
Symptoms	Transient Ischemic Attacks	**Gender**	M
Smoking Status	Ex-smoker	**Age**	69
Medications	Simvastatin, Felate, Quinapril, Warfrin, Thiamine, Omeprazole		

Figure 3.1.1 Sectioning and Zones of Plaque A
Plaque A was removed from the left carotid artery and cut into 9 sections each of which was homogenised. Sections 1-2 were the pre-bifurcation zone, sections 3-4 were the bifurcation zone and sections 5-9 were the post-bifurcation zone. Top right hand insert not to scale.

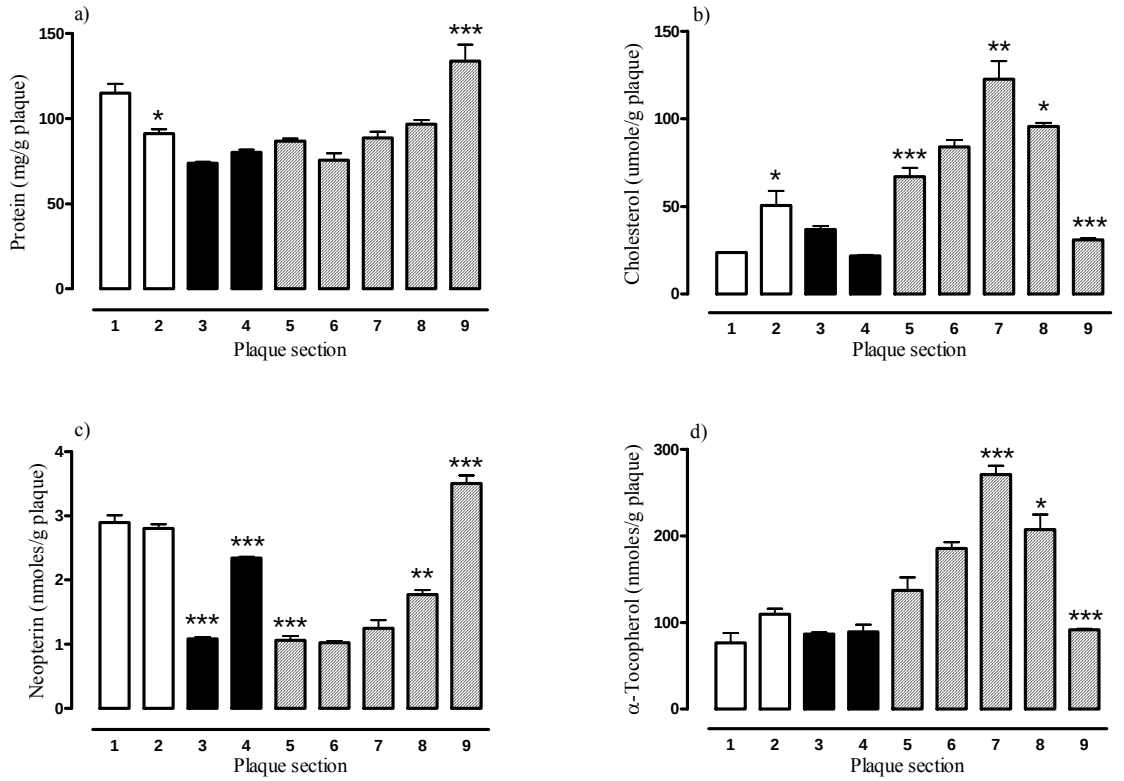

Figure 3.1.2 Protein, Cholesterol, Neopterin and α-Tocopherol Content in Plaque A
Protein (a) and cholesterol (b) concentrations were determined spectrophotometrically, while the neopterin (c) and α-tocopherol (d) were quantified by reverse phase HPLC. The clear bars represent the pre-bifurcation zone, solid bars the bifurcation zone and stripped bars the post-bifurcation zone. Each bar represents the mean ± SEM of the analysis from triplicate samples.

The protein content in plaque A showed a significant decrease between section 1 and 2 ($P<0.05$), it then remained similar throughout the remaining sections of the plaque until there was a large rise in the final section (Figure 3.1.2a). The cholesterol concentration showed a highly significant increase between the bifurcation and post-bifurcation zones ($P<0.001$). The highest level of cholesterol was found in section 7 with a sudden decrease in concentration in sections 8 and 9 (Figure 3.1.2b). There was significant variations in the neopterin concentration along the length of plaque A with decreases ($P<0.001$) in the concentration for the first section of both the bifurcation and post-bifurcation zones (sections 3 and 5 respectively). The highest concentration of neopterin was found in the last section of the plaque (Figure 3.1.2c). The α-tocopherol concentration followed a similar trend to that of cholesterol with the highest concentration found in section 7 at 270.92 ± 10.38 nmoles/g plaque (Figure 3.1.2d)

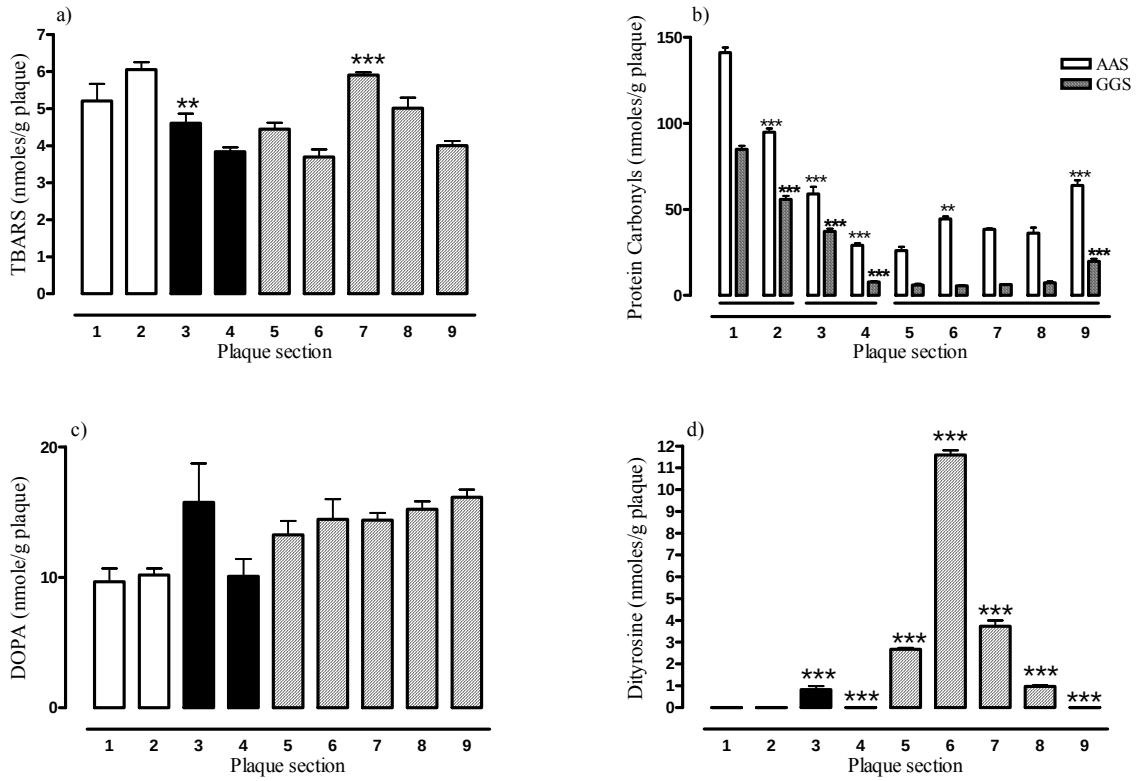

Figure 3.1.3 Lipid and Protein Oxidation Markers in Plaque A
TBARS (a) protein carbonyls AAS and GGS (b) DOPA (c) and dityrosine (d) were all quantified via reverse phase HPLC. Clear bars represent the pre-bifurcation zone, solid bars the bifurcation zone and stripped bars the post-bifurcation zone in graphs (a), (c) and (d). The lines underneath the bars in graph (b) indicate the separate zones. Each bar represents the mean ± SEM of the analysis from triplicate samples.

Plaque A's TBARS content showed a decrease in concentration between the pre-bifurcation and bifurcation zones ($P<0.01$), and a highly significant rise from section 6 to 7 (Figure 3.1.3a). The highest concentration of protein carbonyls AAS and GGS was found in section 1, with significant decreases down in section 2, 3 and 4 ($P<0.001$). Apart from a rise in section 6's AAS, both the protein carbonyls concentrations remained stable throughout the post-bifurcation zone until increasing sharply in section 9 (Figure 3.1.3b). The DOPA concentrations remained at a consistent level across all sections of plaque A (Figure 3.1.3c). There was no dityrosine detectable in the pre-bifurcation zone of plaque A, nor was there any in sections 4 and 9. The majority of dityrosine was found in the post-bifurcation zone with the highest concentration of 11.58 ± 0.22 in section 6 (Figure 3.1.3c). Strong correlations in trends across sections can be seen between cholesterol, α-tocopherol and dityrosine with low concentrations in the pre-bifurcation and bifurcation zones and an increasing concentration in the middle of the post bifurcation zone of all

three markers. A significant decrease in concentration between the pre-bifurcation and bifurcation zone occurred in four of the eight markers analysed (neopterin, TBARS, AAS and GGS protein carbonyls). Four markers (protein, neopterin, AAS and GGS protein carbonyls) also had a significant increase in the concentration of section 9.

3.1.2 Plaque B (Plaque Laboratory No 19)

Plaque B was removed from the left carotid artery. It was characterised with 75% stenosis, 26 mm in length and weighed a total mass of 0.406 grams. A very clear secondary branch was seen with both openings visible in section 4 (Figure 3.1.4). The majority of the stenosis was seen in the post-bifurcation sections 5 and 6. Plaque B showed no signs of calcification.

Table 3.1.2 Plaque B Patient and Clinical Information

Location	Left Carotid Artery	**Stenosis**	75%
Symptoms	Stoke	**Gender**	M
Smoking Status	Ex-smoker	**Age**	68
Medications	Omeprazole, Enalapril, Pentasa, Aspirin, Simvastatin		

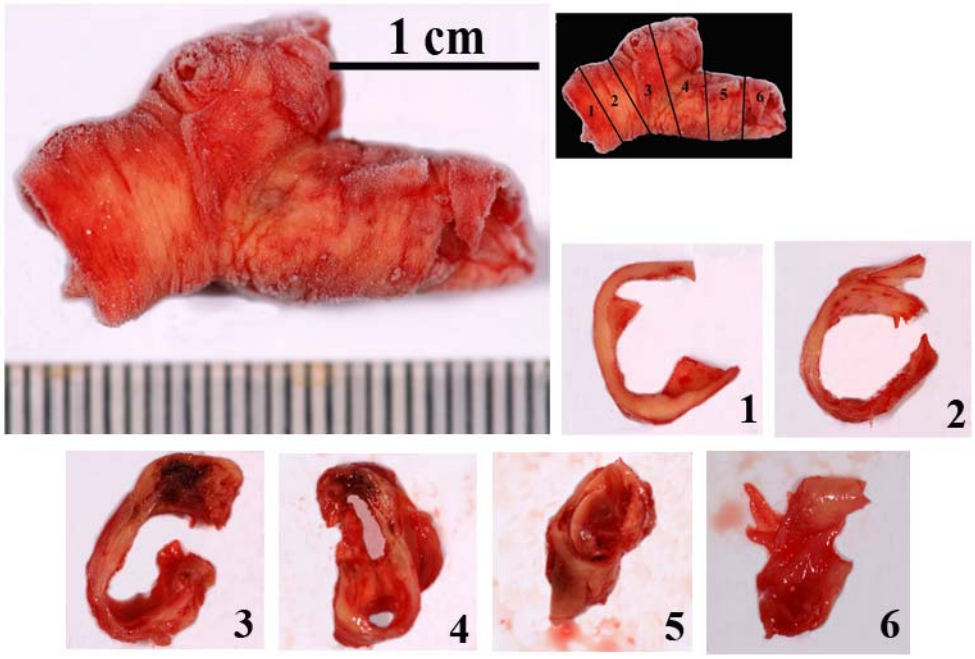

Figure 3.1.4 Sectioning and Zones of Plaque B
Plaque B was removed from the left carotid artery and cut into 6 sections, each of which was homogenised. Sections 1-2 were the pre-bifurcation zone, sections 3-4 were the bifurcation zone and sections 5-6 were the post-bifurcation zone. Top right hand insert not to scale.

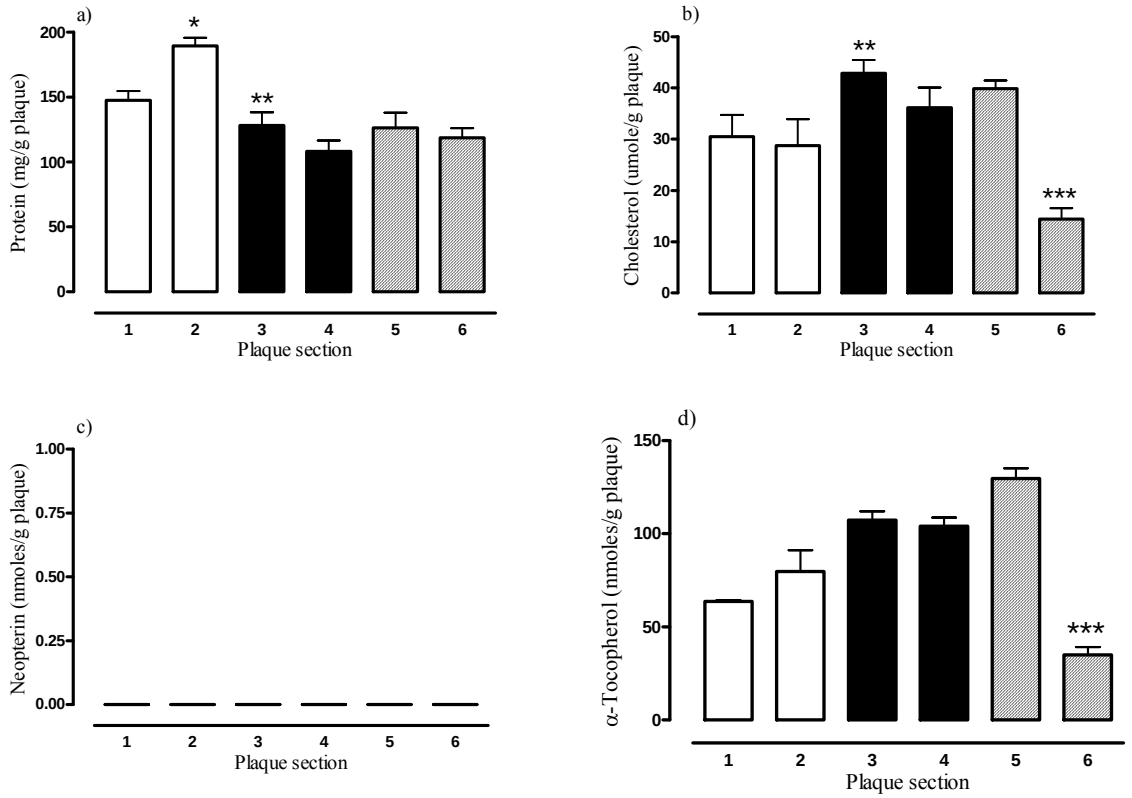

Figure 3.1.5 Protein, Cholesterol, Neopterin and α-Tocopherol Content in Plaque B
Protein (a) and cholesterol (b) concentrations were determined spectrophotometrically, while the neopterin (c) and α-tocopherol (d) were quantified by reverse phase HPLC. The clear bars represent the pre-bifurcation zone, solid bars the bifurcation zone and stripped bars the post-bifurcation zone. Each bar represents the mean ± SEM of the analysis from triplicate samples.

The highest protein concentration in plaque B was in section 2 at 189.49 ± 10.72 mg/g plaque, followed by a significant decrease in the bifurcation zone (Figure 3.1.5a). The cholesterol content showed the opposite trend, there was a significant increase in the concentration between the pre-bifurcation and bifurcation zones, and also a highly significant decrease ($P<0.001$) in the last section (Figure 3.1.5b). Neopterin levels were below the detectable limit in plaque B (Figure 3.1.5c). Although the α-tocopherol content showed no significant variation between neighbouring sections 1 to 5, there did appear to be a general increase in concentration peaking at 129.57 ± 5.49 in section 5, followed by a dramatic decrease in section 6 (Figure 3.1.5d).

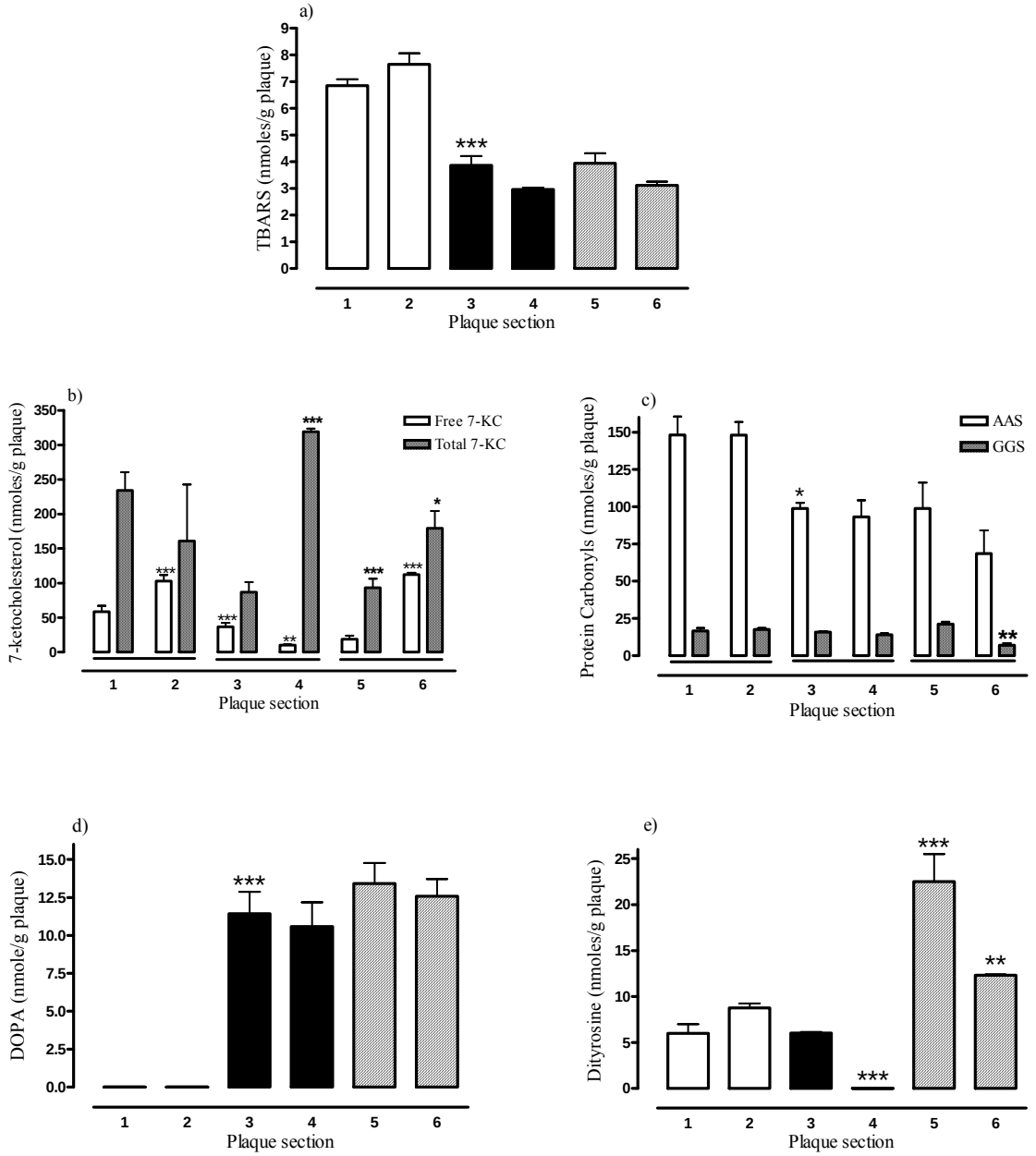

Figure 3.1.6 Lipid and Protein Oxidation Markers in Plaque B
TBARS (a) 7-ketocholesterol (b) protein carbonyls AAS and GGS (c) DOPA (d) and dityrosine (e) were all quantified via reverse phase HPLC. Clear bars represent the pre-bifurcation zone, solid bars the bifurcation zone and stripped bars the post-bifurcation zone in graphs (a), (d) and (e). The lines underneath the bars in graph (b) and (c) indicate the separate zones. Each bar represents the mean ± SEM of the analysis from triplicate samples.

The pre-bifurcation zone of plaque B contained significantly higher amounts of TBARS compared to both the bifurcation and post-bifurcation zones (Figure 3.1.6a). The opposite trend to this was seen in the DOPA content, where DOPA was below the detection limit

in the pre-bifurcation zone and at a steady concentration throughout the rest of the plaque (Figure 3.1.6d). 7-Ketocholesterol showed no correlation between the concentrations of free and total 7-KC, the levels of both varied dramatically across the length of the plaque (Figure 3.1.6b). Both protein carbonyls in plaque B remained at relatively consistent concentrations across the plaque, with the AAS concentrations always significantly higher than the GGS concentrations (Figure 3.1.6c). The dityrosine levels in plaque B were low across sections 1, 2 and 3, dropping below the detection limit in section 4 before they increased significantly in the post-bifurcation zone ($P<0.001$) (Figure 3.1.6e). Trends seen in plaque B showed a significant decrease between the pre-bifurcation and bifurcation zones in protein, TBARS, free 7-KC and AAS protein carbonyl. Section 6 contained the lowest concentration of four of the markers, cholesterol, α-tocopherol, AAS and GGS protein carbonyls, while there was a significant increase between the pre-bifurcation and bifurcation zones of cholesterol and DOPA.

3.1.3 Plaque C (Plaque Laboratory No 21)

Plaque C was removed from the left carotid artery. It was 28 mm in length, weighed total mass of 0.167 grams and had 90% stenosis. There was only a very small secondary branch in section 1 making this the bifurcation section (Figure 3.1.7). All remaining sections were in the post-bifurcation zone. Plaque C had no pre-bifurcation sections, no calcification and no thrombus present

Table 3.1.3 Plaque C Patient and Clinical Information

Location	Left Carotid Artery	**Stenosis**	90%
Symptoms	Stoke	**Gender**	F
Smoking Status	Non-smoker	**Age**	63
Medications	Aspirin, Cilazapril, Simvastatin, Fluticasone inhaler		

Figure 3.1.7 Sectioning and Zones of Plaque C
Plaque C was removed from the left carotid artery and cut into 4 sections, each of which was then homogenised. Section 1 was the bifurcation zone and sections 2-4 were the post-bifurcation zone. Top right hand insert not to scale

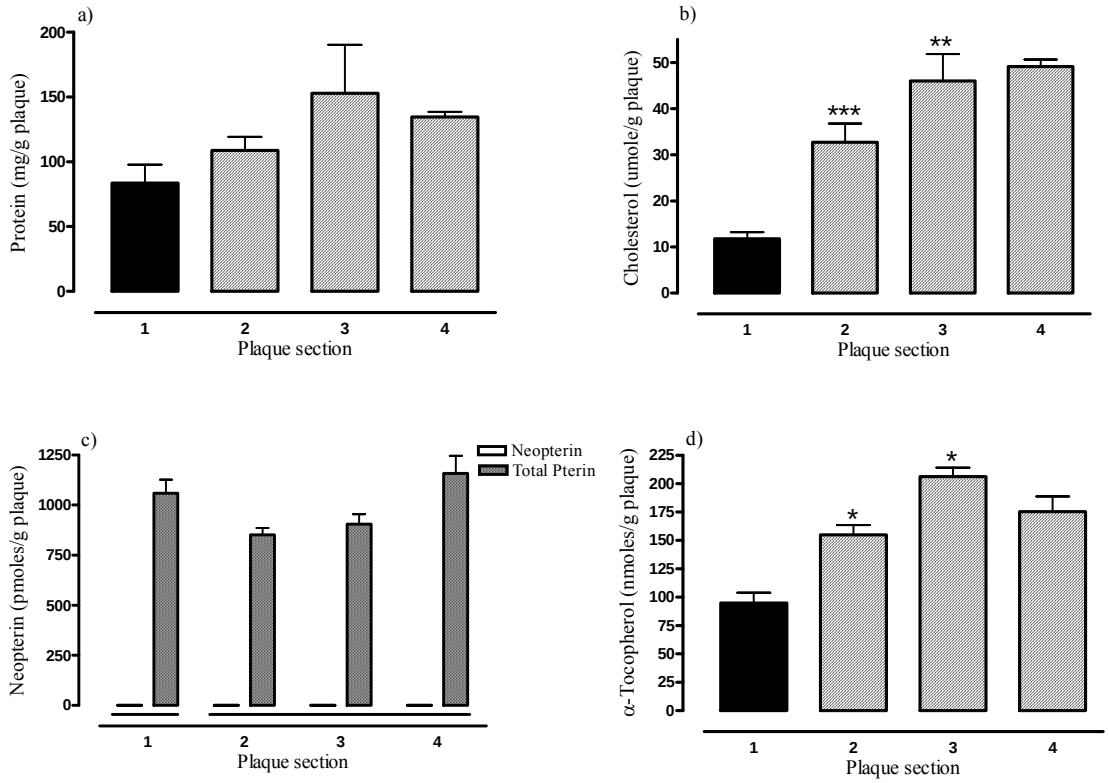

Figure 3.1.8 Protein, Cholesterol, Neopterin and α-Tocopherol Content in Plaque C
Protein (a) and cholesterol (b) concentrations were determined spectrophotometrically, while the neopterin (c) and α-tocopherol (d) were quantified by reverse phase HPLC. In graphs (a), (b) and (d) clear bars represent the pre-bifurcation zone, solid bars the bifurcation zone and stripped bars the post-bifurcation zone. The lines underneath the bars in graph (c) indicate the separate zones. Each bar represents the mean ± SEM of the analysis from triplicate samples.

The protein content showed no significant changes between neighbouring sections of plaque C and had an average concentration of 119.93 ± 16.41 mg/g plaque (Figure 3.1.8a). The bifurcation zone contained a significantly lower cholesterol content then the post-bifurcation zone ($P<0.001$) (Figure 3.1.8b). Neopterin was undetectable in plaque C but the total pterin content was constant throughout the length of the plaque, indicating that only 7,8-dihydroneopterin was present in this plaque at an average concentration of 993.42 ± 59.95 pmoles/g plaque (Figure 3.1.8c). Plaques C's α-tocopherol content showed a similar trend to that of cholesterol, with the bifurcation section having a significantly lower concentration (Figure 3.1.8d).

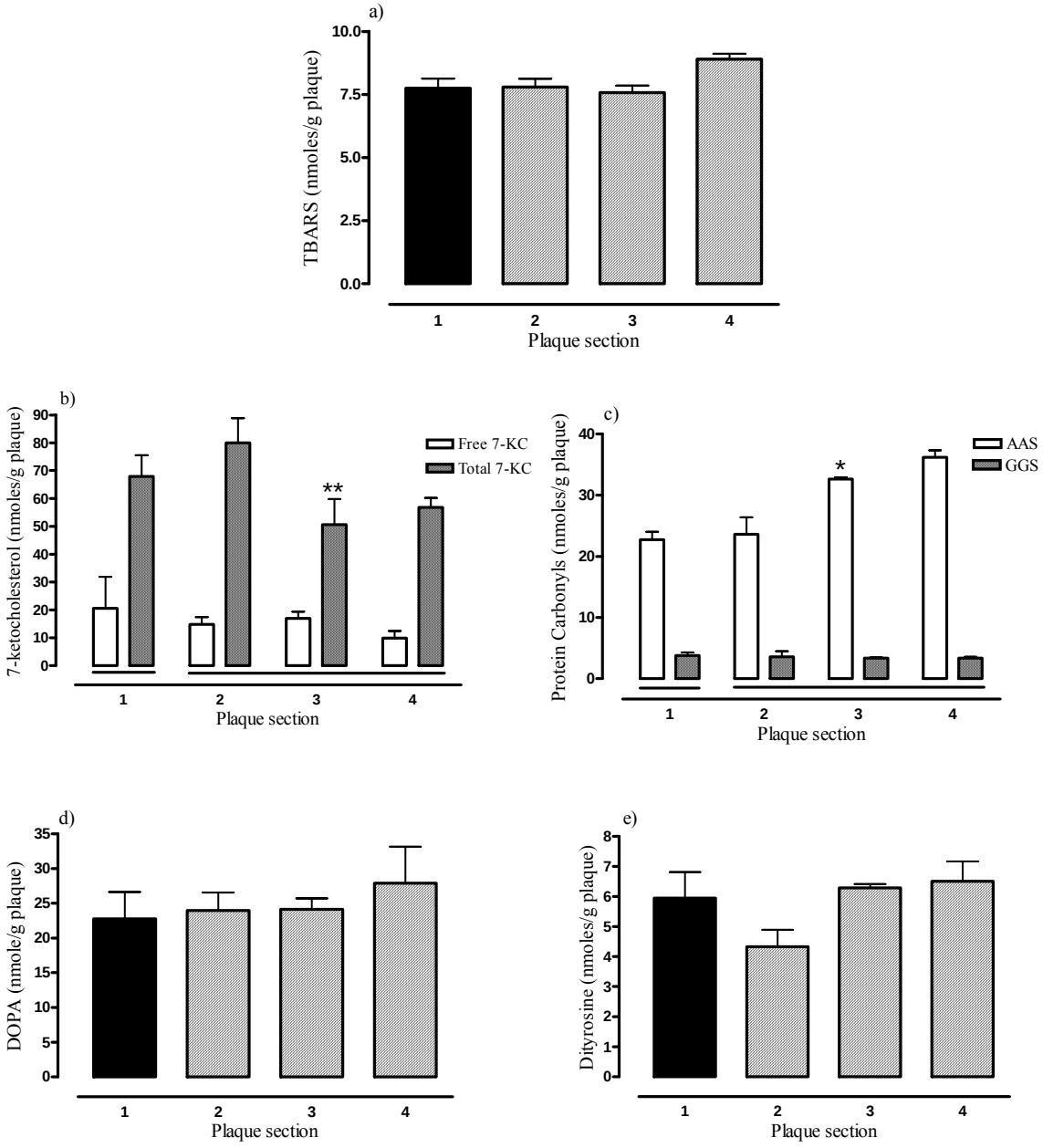

Figure 3.1.9 Lipid and Protein Oxidation Markers in Plaque C
TBARS (a) 7-ketocholesterol (b) protein carbonyls AAS and GGS (c) DOPA (d) and dityrosine (e) were all quantified via reverse phase HPLC. In graphs (a), (d) and (e) clear bars represent the pre-bifurcation zone, solid bars the bifurcation zone and stripped bars the post-bifurcation zone. The lines underneath the bars in graphs (b) and (c) indicate the separate zones. Each bar represents the mean ± SEM of the analysis from triplicate samples.

The TBARS concentration remained constant across all sections of plaque C (Figure 3.1.9a). Although the free 7-ketocholesterol levels in the plaque did not vary

significantly, there was a decrease in concentration of total 7-ketocholesterol between sections 2 and 3 resulting in the last two sections of plaque C having lower total 7-KC (Figure 3.1.9b). The protein carbonyls showed a small rise in AAS content in sections 3 and 4 but no change in the GGS concentrations across the plaque sections (Figure 3.1.9c). Both DOPA and dityrosine had no statistical variation in their content across the plaque, with average concentrations of 24.69 ± 3.31 nmoles/g plaque and 5.76 ± 0.55 nmoles/g plaque respectively (Figure 3.1.9d and Figure 3.1.9e). With the exceptions of cholesterol and α-tocopherol that had significantly lower concentrations in the bifurcation zone, Plaque C showed a stable and consistent distribution in most of the markers across all sections. This indicates no chemical differences between the bifurcation and post-bifurcation zones within this plaque.

3.1.4 Plaque D (Plaque Laboratory No 23)

Plaque D was removed from the right carotid artery. It was characterised with 60 – 79% stenosis, 31 mm in length and weighed a total mass of 0.536 grams. In overall composition, this plaque had a small secondary branch, substantial fatty 'gruel' deposits in section 4, 5 and 6, as well as very small areas of calcification along the plaque (Figure 3.1.10).

Table 3.1.4 Plaque D Patient and Clinical Information

Location	Right Carotid Artery	**Stenosis**	60 - 79%
Symptoms	Stoke	**Gender**	M
Smoking Status	Non-smoker	**Age**	70
Medications	Aspirin, Metoprolol, Simvastatin, Omeprazole, Dipyridamole, Cilazapril		

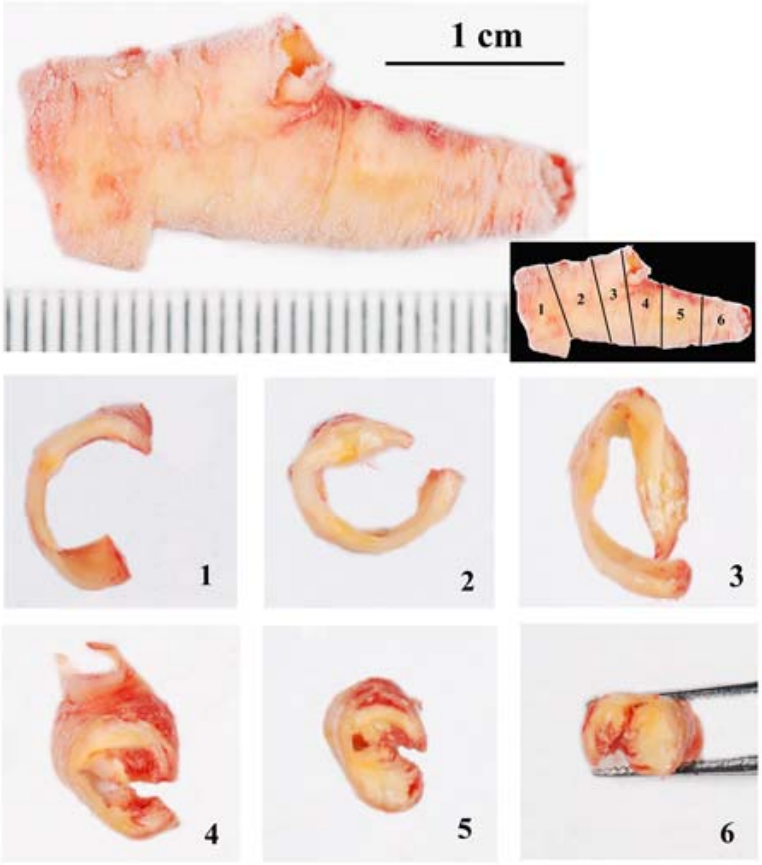

Figure 3.1.10 Sectioning and Zones of Plaque D
Plaque D was removed from the right carotid artery and cut into 6 sections, each of which was homogenised. Sections 1-2 were the pre-bifurcation zone, sections 3-4 were the bifurcation zone and sections 5-6 were the post-bifurcation zone. Top right hand insert not to scale.

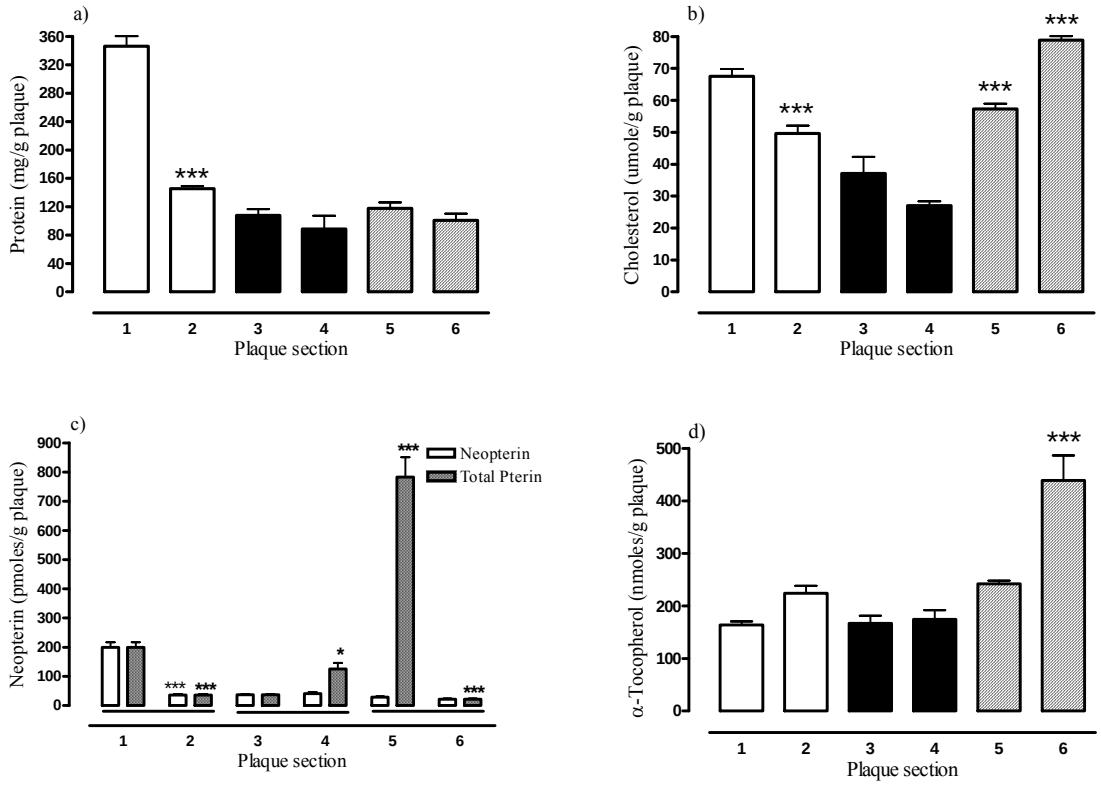

Figure 3.1.11 Protein, Cholesterol, Neopterin and α-Tocopherol Content in Plaque D

Protein (a) and cholesterol (b) concentrations were determined spectrophotometrically, while the neopterin (c) and α-tocopherol (d) were quantified by reverse phase HPLC. In graphs (a), (b) and (d) clear bars represent the pre-bifurcation zone, solid bars the bifurcation zone and stripped bars the post-bifurcation zone. The lines underneath the bars in graph (c) indicate the separate zones. Each bar represents the mean ± SEM of the analysis from triplicate samples.

The highest concentration of protein in plaque D was in section 1 (346.49 ± 13.80 mg/g plaque) followed by a highly significant decrease in section 2 and across the remaining sections of the plaque (Figure 3.1.11a). Plaque D's cholesterol content showed a general trend downwards from section 1 to 4, with the concentration increasing significantly ($P<0.001$) in both sections of the post-bifurcation zone (Figure 3.1.11b). Neopterin levels were significantly high in section 1 at 199.75 ± 17.05 pmoles/g plaque, the concentration remained at a lower level throughout the rest of the plaque. The total pterin levels were only greater than the neopterin levels in section 4 and 5, indicating that 7,8-dihydroneopterin was only present in these two sections. Section 5, the first post-bifurcation section, contained a vast amount of total pterin (782.67 ± 68.50 pmoles/g plaque) compared to the other sections (Figure 3.1.11c). The α-tocopherol content in

plaque D remained at a constant level over the first 5 sections but increased significantly ($P<0.001$) in the last section (Figure 3.1.11d)

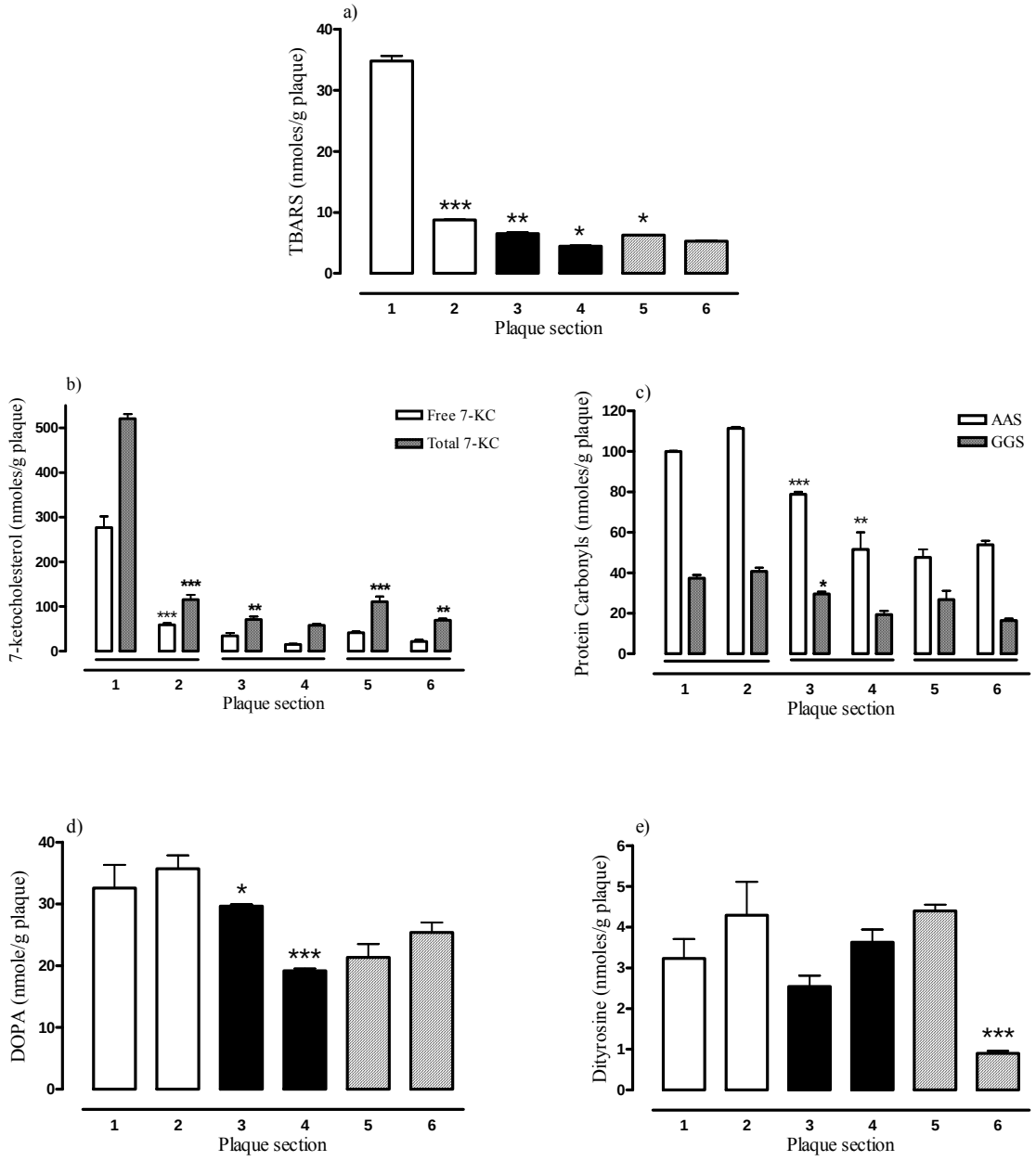

Figure 3.1.12 Lipid and Protein Oxidation Markers in Plaque D
TBARS (a) 7-ketocholesterol (b) protein carbonyls AAS and GGS (c) DOPA (d) and dityrosine (e) were all quantified via reverse phase HPLC. In graphs (a), (d) and (e) clear bars represent the pre-bifurcation zone, solid bars the bifurcation zone and stripped bars the post-bifurcation zone. The lines underneath the bars in graphs (b) and (c) indicate the separate zones. Each bar represents the mean ± SEM of the analysis from triplicate samples.

The TBARS content showed a similar trend to that of the protein content in plaque D with section 1 containing a significantly higher concentration than the rest of the plaque

(Figure 3.1.12a). Section 1 was also significantly high ($P<0.001$) in both free and total 7-KC content, the concentration decreased in section 2 and remained lower across the rest of the plaque. There was an increase in total 7-KC concentration between the bifurcation and post-bifurcation zones (Figure 3.1.12b). Both protein carbonyls AAS and GGS had a significant decrease in concentration from the pre-bifurcation zone to the bifurcation zone (Figure 3.1.12c). This decrease in concentration at the bifurcation zone was also seen in the DOPA content of plaque D (Figure 3.1.12d). There was no significant changes in the dityrosine concentration between sections 1 to 5 but it decreased significantly ($P<0.001$) in section 6 (Figure 3.1.12e). The major trends seen in this plaque were a significant decrease in concentration between sections 1 and 2 occurring in a total of six markers (protein, cholesterol, neopterin, TBARS, free 7-KC and total 7-KC). All the oxidation markers (Figure 3.1.12) showed a decrease in concentration between the pre-bifurcation and bifurcation zones, while both cholesterol and α-tocopherol had a significant increase in their concentrations at the post-bifurcation zone.

3.1.5 Plaque E (Plaque Laboratory No 23)

Plaque E was from a right carotid artery. It was 30 mm in length, weighed a total mass of 0.914 grams and had 60% stenosis. This plaque had a large secondary branch which covered three sections, while no no calcification was found all sections contained large amounts of 'gruel' (Figure 3.1.13).

Table 3.1.5 Plaque E Patient and Clinical Information

Location	Right Carotid Artery	**Stenosis**	60%
Symptoms	Transient Ischemic Attacks	**Gender**	M
Smoking Status	EX-smoker	**Age**	73
Medications	Aspirin, Dipyridamole, Quinapril, Accuretic, Ezetimibe, Felodipine ER, Atenolol, Protophane, Novorapid		

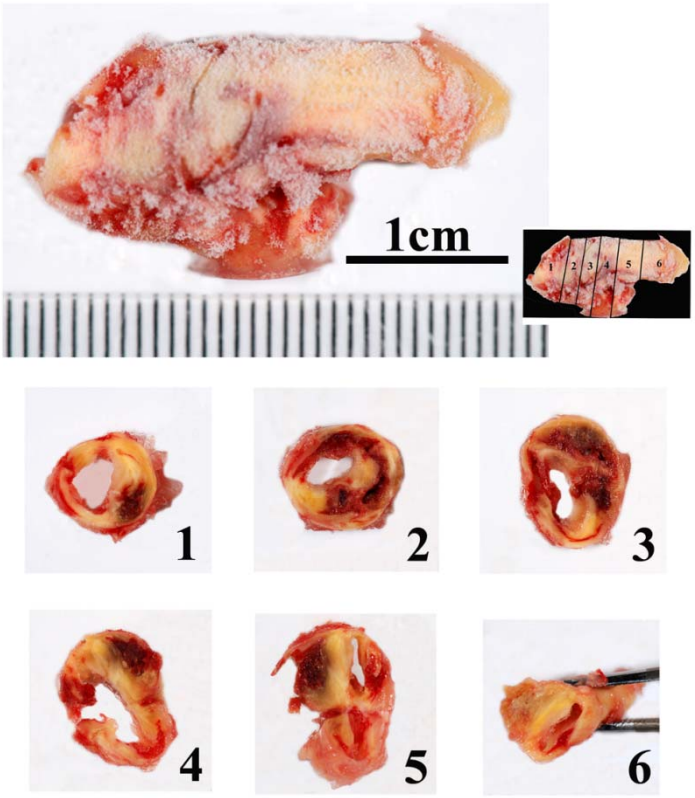

Figure 3.1.13 Sectioning and Zones of Plaque E
Plaque E was removed from the right carotid artery and cut into 6 sections, each of which was homogenised. Sections 1-2 were the pre-bifurcation zone, sections 3-5 were the bifurcation zone and section 6 was the post-bifurcation zone. Top right hand insert not to scale.

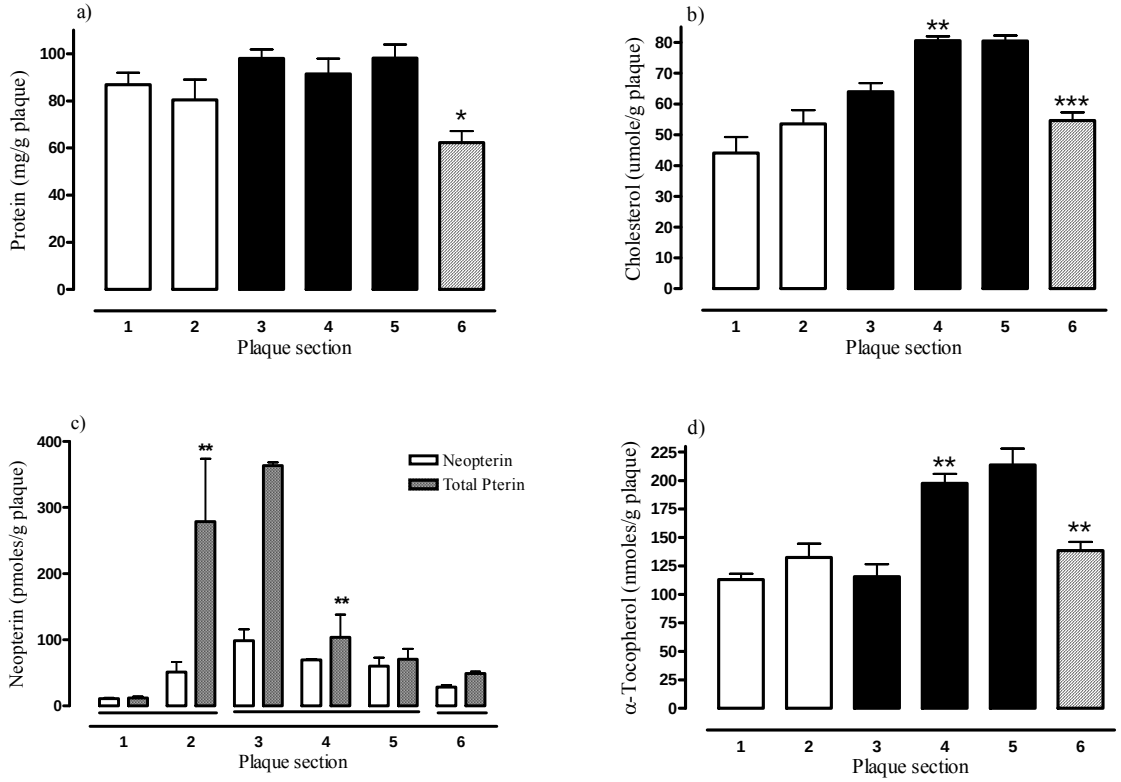

Figure 3.1.14 Protein, Cholesterol, Neopterin and α-Tocopherol Content in Plaque E

Protein (a) and cholesterol (b) concentrations were determined spectrophotometrically, while the neopterin (c) and α-tocopherol (d) were quantified by reverse phase HPLC. In graphs (a), (b) and (d) clear bars represent the pre-bifurcation zone, solid bars the bifurcation zone and stripped bars the post-bifurcation zone. The lines underneath the bars in graph (c) indicate the separate zones. Each bar represents the mean ± SEM of the analysis from triplicate samples.

Plaque E's protein content was consistent throughout most sections of the plaque with only a decrease in concentration in the post-bifurcation zone (Figure 3.1.14a). The cholesterol content in this plaque showed a general trend of increasing concentration in the pre-bifurcation and bifurcation zones, although the only significant increase in concentration was in section 4. There was also a significant decrease in the cholesterol content in the post-bifurcation zone (Figure 3.1.14b). The total pterin concentrations were significantly higher in section 2 and 3, reaching the highest concentration of 363.63 ± 4.75 pmoles/g plaque in section 3. Section 3 contained the highest concentration of neopterin as well (Figure 3.1.14c). α-Tocopherol showed a very similar trend to cholesterol, with a significant increase in concentration between sections 3 and 4, and again a decrease in concentration in section 6, the post-bifurcation zone (Figure 3.1.14d)

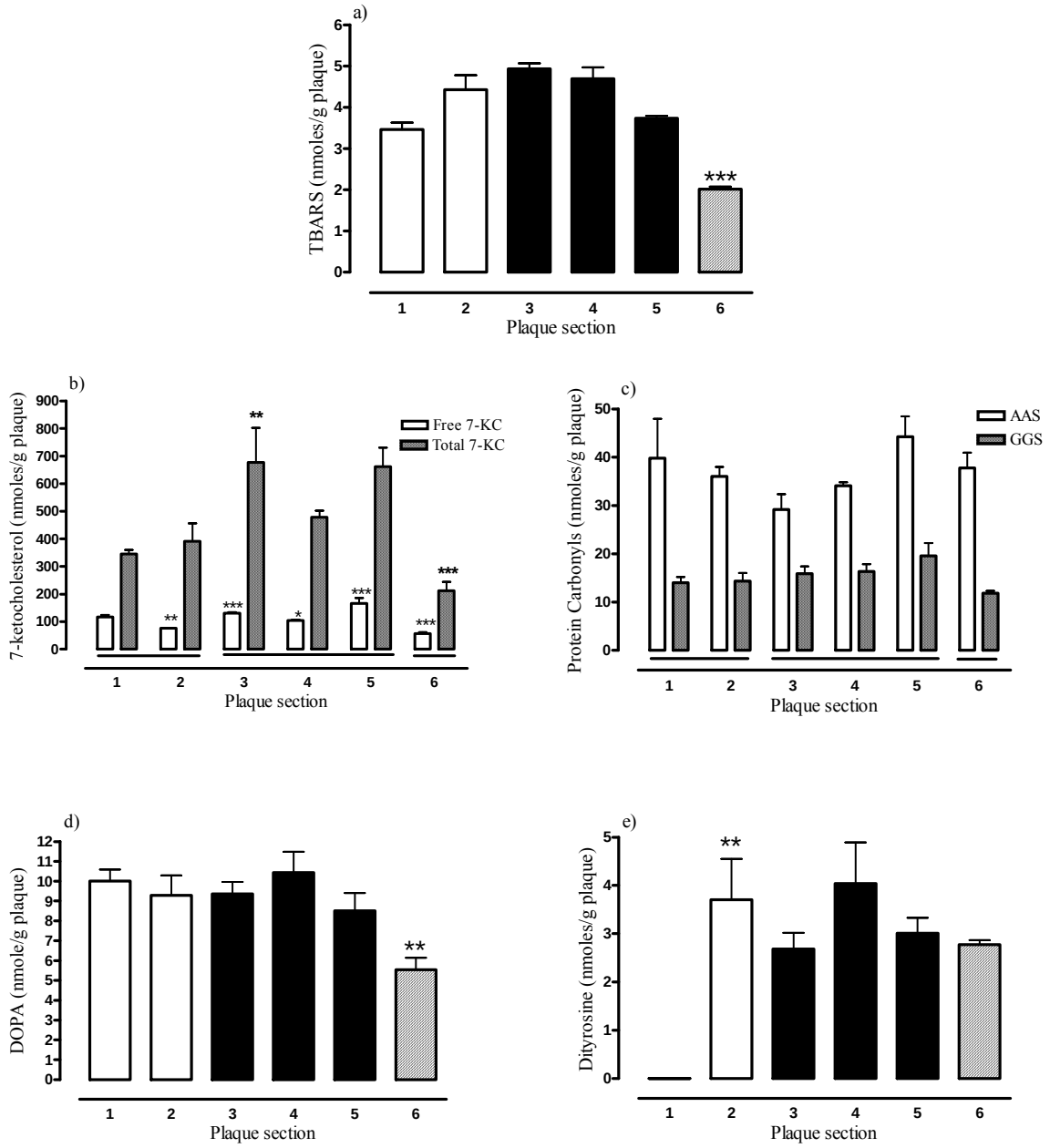

Figure 3.1.15 Lipid and Protein Oxidation Markers in Plaque E
TBARS (a) 7-ketocholesterol (b) protein carbonyls AAS and GGS (c) DOPA (d) and dityrosine (e) were all quantified via reverse phase HPLC. In graphs (a), (d) and (e) clear bars represent the pre-bifurcation zone, solid bars the bifurcation zone and stripped bars the post-bifurcation zone. The lines underneath the bars in graphs (b) and (c) indicate the separate zones. Each bar represents the mean ± SEM of the analysis from triplicate samples.

Both TBARS and DOPA have a similar trend to the protein content (Figure 3.1.14a) in plaque E as they all have a relatively consistent levels across the first five sections of the plaque before a significant decrease in concentration of section 6 (Figures 3.1.15a and

3.1.15d). The free 7-KC had variable concentration across the length of the plaque, showing significant increases ($P<0.001$) in sections 3 and 5. There was also an increase in the total 7-KC concentration in section 3, as well as a significant drop in concentration for both of the markers in the post-bifurcation zone (Figure 3.1.15c). AAS and GGS both remained at consistent concentrations across all sections of plaque E. AAS gave an average concentration of 36.87 ± 3.55 nmoles/g plaque and GSS 15.32 ± 1.51 nmoles/g plaque (Figure 3.1.15c). The dityrosine content was below the detectable limit in section 1 but remained at a consistent concentration throughout the remaining sections of plaque E (Figure 3.1.15e). A total of seven markers (protein, cholesterol, α-tocopherol, TBARS, free 7-KC, total 7-KC and DOPA) showed a significant decrease in the post-bifurcation zone, section 6. But with the exception of 7-KC and total pterin there was not a lot of variation between the previous five sections across the length of this plaque.

3.1.6 Correlations between Markers of Individual Plaques

Correlation statistics were applied to all possible pairs of markers in each plaque, with all significant positive and negative correlations displayed in Tables 3.1.6. and 3.1.7. Although there was a large amount of variation in marker correlation, highlighting the differences between and within the plaques, some correlations occurred twice or more among the five plaques (A-E).

Only one correlation occurred in all five plaques, a positive correlation between α-tocopherol vs. cholesterol which was highly significant in all occasions (Table 3.1.6). This correlation was also significant in four of the previously analysed plaques (Table 3.1.7). The positive correlation between AAS and GGS protein carbonyls was significant in four of the five plaques (plaques A, B, C and D). Another two major correlations with three out of the five plaques having a positive correlations were between protein vs. TBARS, and protein vs. free 7-KC. Many correlations did occur twice with some pairs of plaques sharing more than on set of correlations. Plaques A and D both shared two negative correlations between protein vs. dityrosine and neopterin vs. dityrosine, the negative neopterin and dityrosine correlation was found in two more of the previously analysed plaques (Table 3.1.7). Plaques D and E both shared a vast seven positive correlations together between neopterin vs. protein, neopterin vs. TBARS, DOPA vs.

TBARS, total 7-KC vs. protein, total 7-KC vs. neopterin, total 7-KC vs. TBARS and total 7-KC vs. free 7-KC (Table 3.1.7).

Table 3.1.6 Correlation of Inflammatory and Oxidative Markers in Plaques A-E
Significant correlations were determined based on the average of each section (n=3) within plaques A-E using Statistica's correlation matrices. (+) Indicates a positive correlation, (-) indicates a negative correlation. Letter abbreviations in table correspond to the plaque assigned letters. Extended table in Appendix. Statistical significance represented by P<0.05 * P<0.01 ** P<0.001 ***

	Pro	Chol	Neop	α-toc	TBARS	DOPA	Dityr	AAS	GGS	Free 7-KC
Chol	-A* +C**									
Neop	+D*** +E*	-A*** +E*								
α-toc		+A*** +B*** +C** +D** +E***	-A*							
TBARS	+B*** +D*** +E*		+D*** +E**							
DOPA	-B* +D**		-A* +D*		-B*** +D** +E**					
Dityr	-A* -D** +E*	+A***	-A** -D**	+A**	-D*** +E*	+E**				
AAS	+A** +B**	-A** +C**	+A***	-A* +C**	+B***	-B***				
GGS	+E*	-A**	+A***	-A** +B*	+A*	-A*	-A* +E*	+A*** +B** +C* +D***		
Free 7-KC	+B* +D*** +E**	+E*	+D***		+D***	+D*	-D*** +E*			
Total 7-KC	+D*** +E**	+E*	+D*** +E**		+D*** +E*	+C** +D**	-D***		+E*	+D*** +E**

Pro – Protein, Chol – Cholesterol, Neop – Neopterin, α-toc - α-tocopherol, Dityr – Dityrosine, AAS – AAS protein carbonyl, GGS – GGS protein carbonyl, 7-KC – 7-ketocholesterol

Table 3.1.7 Correlation of Inflammatory and Oxidative Markers in Plaques A-S
Significant correlations were determined based on the average of each section (n=3) within plaques A-S. Plaques A-E were analysed in this study using Statistica's correlation matrices Plaques H-M were analysed by Flavall (2008) and plaques N-S by Crone (2008). (+) Indicates a positive correlation, (-) indicates a negative correlation. Letter abbreviations in table correspond to the plaque assigned letters. Statistical significance represented by P<0.05 * P<0.01 ** P<0.001 ***

	Pro	Chol	Neop	α-toc	TBAR	DOPA	Dityr	AAS	GGS	Free 7-KC
Chol	-A* +C** -K* +R*									
Neop	+D*** +E* +J* +L** +N***	-A*** +E*								
α-toc	+N*	+A*** +B*** +C** +D** +E*** +H*** +L** +P* +R***	-A* +N*							
TBARS	+B*** +D*** +E* +R*	+M* +R**	+D*** +E** +M* +N*	+J* +R*						
DOPA	-B* +D** +Q**	+M* -P**	-A* +D*	-P*	-B*** +D** +E** -Q*					
Dityr	-A* -D** +E* +H* +Q*	+A*** +I* +P*	-A** -D** -K* -P*	+A** -Q*	-D*** +E*	+E**				
G C P	+L*	-K*	+L*	+I* +N**	+K* +N**	+O*				
AAS	+A** +B**	-A** +C**	+A***	-A* +C**	+B***	-B***				
GGS	+E*	-A**	+A***	-A** +B*	+A*	-A*	-A* +E*	+A*** +B** +C* +D***		
Free 7-KC	+B* +D*** +E**	+E*	+D***		+D***	+D*	-D*** +E*			
Total 7-KC	+D*** +E**	+E*	+D*** +E**		+D*** +E*	+C** +D**	-D***		+E*	+D*** +E**

Pro – Protein, Chol – Cholesterol, Neop – Neopterin, α-toc - α-tocopherol, Dityr – Dityrosine, G C P – General Protein Carbonyls, AAS – AAS protein carbonyl, GGS – GGS protein carbonyl, 7-KC – 7-ketocholesterol

3.1.7 Combined Plaque Analysis

Due to a small sample set (N=5) and the complexity of the plaques, correlations within the individual plaques (A-E) occurred between almost all the pairs of markers (Table 3.1.6 and Figure 3.1.16a). Using a higher number of plaque samples it is more likely that common factors and correlations will emerge. This research was a continuation from a PhD (Firth, 2006) and two masters (Crone, 2008; Flavall, 2008) research which has collectively analysed some of the same markers within thirteen plaques. To better understand the processes occurring within these plaques the entire eighteen plaques were statistically analysed to identify any underlying trends within the inflammatory and oxidative markers.

All of the eighteen plaques were in an advanced stage of the disease with stenosis between 60-90%. The patients presented with a range of symptoms including stroke, transient ischemic attack (very short lived occlusion of artery in brain), and amaurosis fugax (temporary loss of vision) (Table 3.1.9, Figure 3.1.16 and Figure 3.1.17). The plaques were classified by there difference in morphological shapes, being either in the form of a Y shape where both branches were of similar size, had a smaller secondary side branch, or was linear with no side protrusions. Another qualitative characteristic assessed during homogenisation was the overall composition of the plaque, this was classified as being either calcified, contained an obvious thrombus or did not strongly have either of these features (Table 3.1.8).

Table 3.1.8 Summary of Clinical and Patient Information in Plaques A-S

Plaque	Total Mass (g)	Morp.	Composition	Section N	Location				Symptoms
					LC	RC	LF	RF	
A	1.179	Small	Neither	9	*				TIA
B	0.406	Y	Neither	6	*				Stroke
C	0.167	Linear	Neither	4	*				Stroke
D	0.536	Small	Calcified	6		*			Stroke
E	0.914	Small	Neither	6		*			TIA
F	0.909	Linear	Neither	6		*			TIA
G	2.857	Linear	Calcified	7		*			Stroke
H	1.500	Small	Neither	8		*			TIA
I	0.763	Small	Thrombosis	7	*				Stroke
J	1.306	Small	Calcified	7	*				TIA
K	0.822	Y	Thrombosis	6		*			AF
L	0.597	Y	Calcified	6	*				Stroke
M	0.377	Small	Thrombosis	6	*		*		AF
N	1.156	Y	Calcified	8				*	Unknown
O	0.472	Linear	Calcified	3					Unknown
P	0.814	Linear	Neither	7	*				TIA
Q	1.238	Y	Thrombosis	7		*			Stroke
R	0.943	Small	Thrombosis	8		*			Asymptom
S	0.692	Linear	Unknown	Whole		*			TIA

Overall morphology (Morp.): Y – Y shaped, Small – small secondary branch and linear.
Overall composition (Composition):Thrombosed, calcified or neither.
Section: number of sections each plaque was homogenised into.
Arterial location in plaque: LC – left carotid, RC – right carotid, LF – left femoral, and RF – right femoral.
Patient Symptoms: TIA – transient ischemic attack, AF – amaurosis fugax, Stroke, and asymptomatic.

3.1.8 Correlations for the Combined Plaque Data

Combined the eighteen plaques still showed a positive correlation between cholesterol and α-tocopherol (Table 3.1.9). α-Tocopherol also positively correlated with protein and general protein carbonyls (only analysed in plaques F-S). However α-tocopherol did not correlate to either of the specific protein carbonyls AAS and GGS (analysed in plaques A-E).

The general protein carbonyls (F-S) positively correlated to protein and neopterin, although both specific protein carbonyls AAS and GGS showed only a very high statistical correlation to protein ($P<0.001$), but no correlation to neopterin. AAS and GGS both had positive correlations with TBARS, free 7-KC and each other (Table 3.1.8). This difference in the correlations of the general protein carbonyls and AAS and GGS protein carbonyls may be due to the specificity of the new protein carbonyl assay, or because only five plaques (A-E) have been analysed for AAS and GGS.

A significant correlation within the individual plaques was between protein vs. TBARS (Table 3.1.6) with three of the five plaques (B, D and E) showing a positive correlation did not show a correlation when all the eighteen plaques data was combined, as only one other plaque (R) also showed this correlation (Table 3.1.7). Three significant positive correlations were observed in the combined plaque analysis that was not seen in any of the individual plaque correlations. These were α-tocopherol vs. protein ($P<0.001$), free 7-KC vs. AAS protein carbonyl ($P<0.05$) and free 7-KC vs. GGS protein carbonyl ($P<0.001$). This difference in marker correlations between the individual plaques and the combined plaque data further supports the diversity among atherosclerotic plaques.

Table 3.1.9 Combined Correlation of Inflammatory and Oxidative Markers between Plaques A-S

Combined plaque analysis was preformed on plaques A-N, P-R. The N column is the collated number of plaque sections the correlation analysis was preformed on. For neopterin correlations plaques F-I were excluded therefore there are lesser number of sections were analysed. For AAS and GGS protein carbonyls correlations only data from plaques from A-E (a total of 31 sections) was analysed, and for 7-ketocholesterol correlations only data from plaques B-E (a total of 22 sections) was analysed. Protein was expressed in mg/g plaque, cholesterol μmole/g plaque and neopterin, α-tocopherol, TBARS, DOPA, Dityrosine, AAS and GGS protein carbonyls and 7-ketocholesterol as nmole/g plaque. Statistical significance represented by P<0.05 * P<0.01 ** P<0.001 ***

Markers	R value	P value		N (sections)
Combined data analysis				
α-tocopherol vs. Protein	0.4135	0.0002	***	114
α-tocopherol vs. Cholesterol	0.2137	0.0043	**	114
α-tocopherol vs. Protein carbonyls	0.2804	0.0091	**	87
DOPA vs. Protein	0.2134	0.0204	*	114
DOPA vs. Dityrosine	0.2634	0.0042	**	114
TBARS vs. Neopterin	0.2479	0.0231	*	80
Protein carbonyls vs. Protein	0.3025	0.0042	**	87
Protein carbonyls vs. Neopterin	0.3807	0.0033	**	59
AAS protein carbonyls vs. Protein	0.8210	0.0001	***	31
AAS protein carbonyls vs. TBARS	0.6822	0.0001	***	31
GGS protein carbonyls vs. Protein	0.6471	0.0002	***	31
GGS protein carbonyls vs. TBARS	0.7098	0.0001	***	31
GGS protein carbonyls vs. Dityrosine	-0.3625	0.0453	*	31
GGS protein carbonyls vs. AAS	0.7950	0.0002	***	31
Free 7-ketocholesterol vs. Protein	0.5906	0.0041	**	22
Free 7-ketocholesterol vs. TBARS	0.6144	0.0022	**	22
Free 7-ketocholesterol vs. AAS	0.5199	0.0131	*	22
Free 7-ketocholesterol vs. GGS	0.7048	0.0002	***	22
Total 7-ketocholesterol vs. Cholesterol	0.5483	0.0081	**	22
Total 7-ketocholesterol vs. Free 7-kc	0.7755	0.0003	***	22

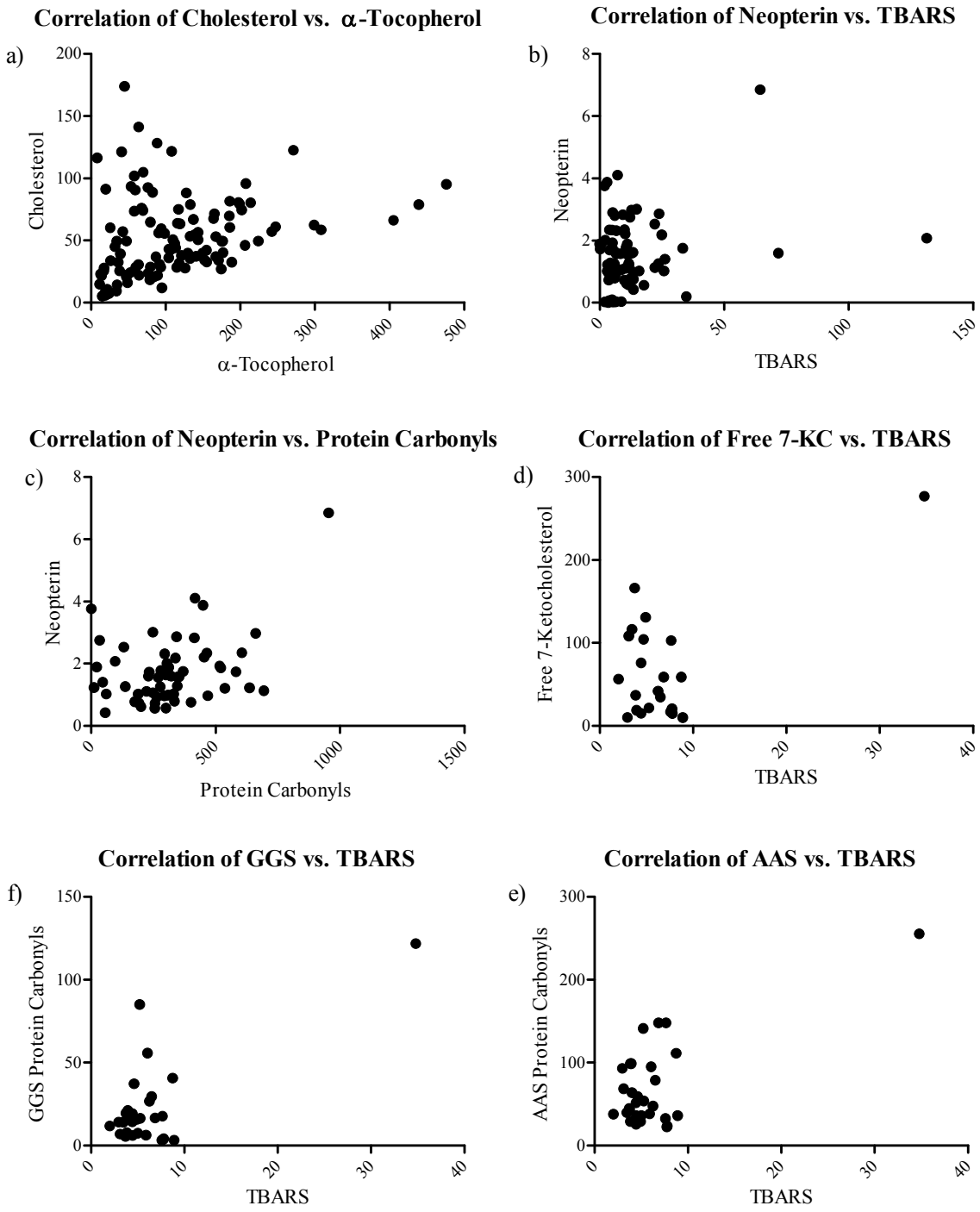

Figure 3.1.16 Correlation Graphs between Interesting Markers for Combined Plaque Data Correlations

All correlation graphs were analysed by Prism (version 5, GraphPad Software, USA).

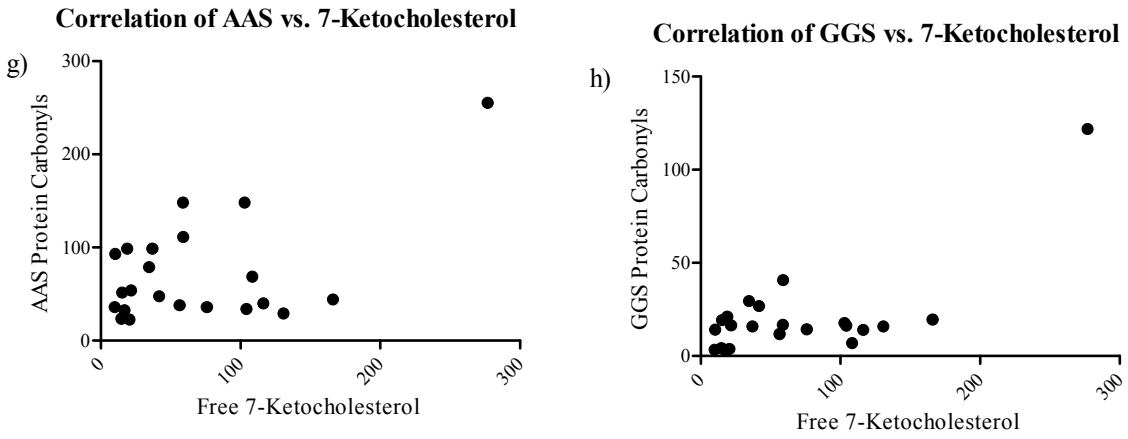

Figure 3.1.17 Correlation Graphs between Interesting Markers for Combined Plaque Data Correlations (2)
All correlation graphs were analysed by Prism (version 5, GraphPad Software, USA).

3.1.9 Variation in Marker Concentration across the Zones

To identify if the concentrations of the inflammatory and oxidative markers varied significantly across the three zones of the plaque, each plaque data was split into the pre-bifurcation, bifurcation and post-bifurcation zones and assessed by one-way ANOVA and Tukey test.

Protein, even though not significant at a p value of 0.096, had a lower concentration in the bifurcation zone (Figure 3.1.18a). The pre-bifurcation zone of cholesterol was on the verge of being significantly lower (P=0.0503) than the bifurcation and post-bifurcation zones (Figure 3.1.18b). Neopterin's concentration was the higher in the pre-bifurcation zone, although not significantly, than the bifurcation zone (P = 0.078), with α-tocopherol exhibiting the opposite trend with its highest concentration in the post-bifurcation zone (P=0.22) (Figures 3.1.18c and 3.1.18d). The concentration of DOPA does not vary significantly across the three zones of the plaque (Figure 3.1.18e). Where as dityrosine had a similar trend to cholesterol, with the pre-bifurcation zone having a significantly lower concentration (P<0.05) than the bifurcation and post-bifurcation zones (Figure 3.1.18f)

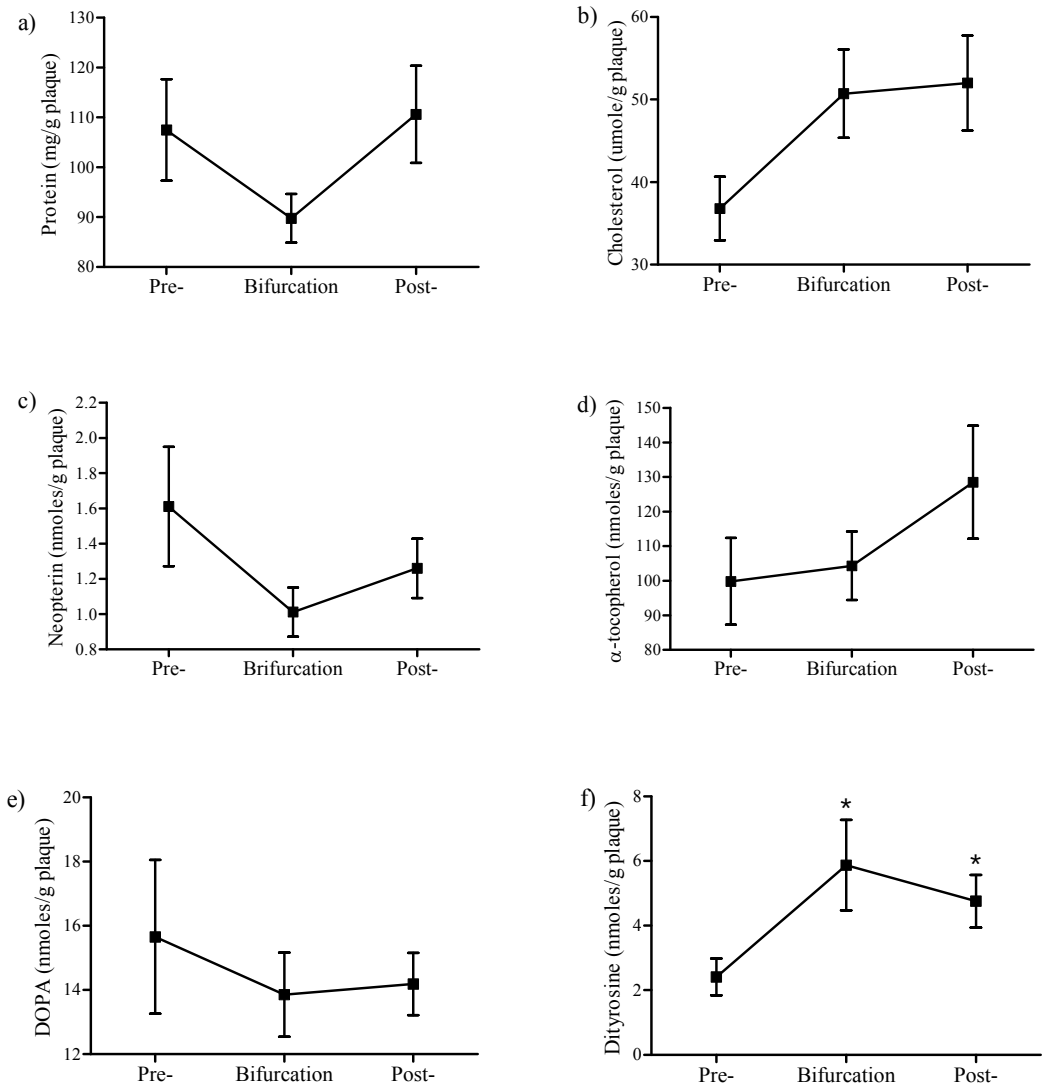

Figure 3.1.18 Effects of Zoning on the Concentration of the Inflammatory and Oxidative Markers within Plaques A-S

Data from each section from plaques A-S (N=18) were classified into either pre-bifurcation (Pre-), bifurcation or post-bifurcation (Post-) zones. The variation between the concentrations of protein (a), cholesterol (b), neopterin (c), α-tocopherol (d), DOPA (e) and dityrosine (f) within each zone was assessed by one way-ANOVA and Tukey's test. Displayed are the means and vertical bars represent the SEM. For the neopterin data only plaques A-E and J-R were included (N=13). Significant differences between zones are represented by $P<0.05$ * $P<0.01$ ** $P<0.001$ ***

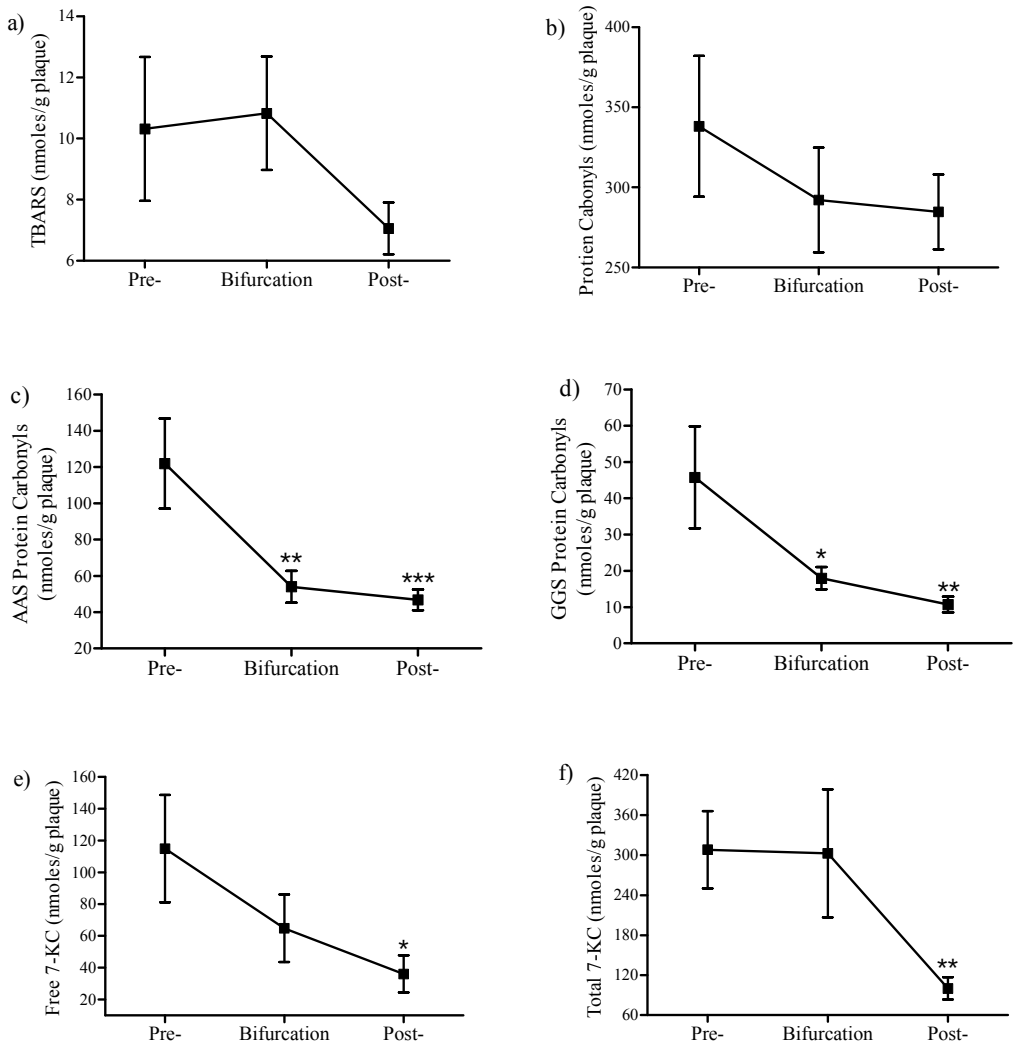

Figure 3.1.19 Effects of Zoning on the Concentration of the Oxidative Markers within Plaques A-S

Data from each section from plaques A-S (N=18) were classified into either pre-bifurcation (Pre-), bifurcation or post-bifurcation (Post-) zones. The variation between the concentrations of TBARS (a), protein carbonyls (b), AAS protein carbonyl (c), GGS protein carbonyl (d), free 7-ketocholesterol (e) and total 7-ketocholesterol (f) within each zone was assessed by one way-ANOVA and Tukey's test. Displayed are the means and vertical bars represent the SEM. For the protein carbonyls data only plaques F-R were included (N=13), for both the AAS and GGS protein carbonyl data only plaques A-E were included (N=5) and for the 7-ketocholesterol data only plaques B-E were included (N=4). Significant differences between zones are represented by $P<0.05$ * $P<0.01$ ** $P<0.001$ ***

TBARS had a high concentration across the pre-bifurcation and bifurcation zones that decreased almost significantly (P=0.056) in the post-bifurcation zone (Figure 3.1.19a). All three types of the protein carbonyls showed a similar trend, with a decrease in the

concentration between the pre-bifurcation and bifurcation zones. Though only the AAS and GGS protein carbonyls had a significant decrease in their concentrations. AAS had a $P<0.01$ and GGS a $P<0.05$ difference between the pre-bifurcation and bifurcation zones. Between the pre-bifurcation and post-bifurcation zones the difference for AAS was $P<0.001$ and GGS was $P<0.01$ (Figures 3.1.19b, 3.1.19c and 3.1.19d). The decrease in the concentration of free 7-ketocholesterol occurs along the length of the plaque with the pre-bifurcation zone containing significantly more than the post-bifurcation zone ($P<0.05$) (Figure3.1.17e). In contrast the total 7-keteocholesterol concentration remains constant in the pre-bifurcation and bifurcation zones before dropping significantly in the post-bifurcation zone ($P<0.01$) (Figure 3.1.17f).

The general zoning trend appears to have high concentrations of protein, cholesterol, α-tocopherol and dityrosine in the post-bifurcation zone of the plaque. With most of the oxidation markers (DOPA, TBARS, protein carbonyls, AAS protein carbonyls, GGS protein carbonyls, free 7-ketocholesterol and total 7-ketocholesterol) being at their highest concentrations in the pre-bifurcation zone and lowest in the post-bifurcation zone. In the pre-bifurcation zone where all the oxidation markers (except dityrosine) are at their highest levels the only anti-oxidant, α-tocopherol, is at its lowest concentration. Yet when all the oxidation markers (except dityrosine) are at their lowest concentrations in the post-bifurcation zone α-tocopherol is at its highest level, indicating that in general the higher the anti-oxidant level in the plaque the lower the amount of oxidation that is occurring.

3.2 Neopterin and 7,8-Dihydroneopterin Concentrations in Human Plasma of CHD Patients Undergoing an Angioplasty

The inflammation marker neopterin has been found within femoral and carotid atherosclerotic plaques (A-S) analysed in our laboratories research, but it has also been found to be elevated in patients with cardio heart disease (Ray et al., 2007; Sahin et al., 2008; Schumacher et al., 1997; Tatzber et al., 1991). By adjusting the previous neopterin assay, using a new Phenomenex reverse phase Synergi 4u Hydro-RP 80A, 250 x 4.6 mm column and increasing the amount of acidic iodide added, the complete conversion of

7,8-dihydroneopterin into the fluoresce neopterin was accomplished which gave the accurate amount of total pterin (neopterin plus 7,8-dihydroneopterin) via HPLC. This new neopterin assay gave the ability to detect both neopterin and 7,8-dihydroneoptin in the plasma of cardio heart disease (CHD) patients.

Our laboratory worked with Dr Catriona Marshall and Dr Dougal McClean from Christchurch Hospital's Department of Cardiology, and obtained a large number of plasma samples from CHD patients. These samples came from two different groups of patients, one group with acute ST-elevation myocardial infarction (STEMI) (N=24), the other group with chronic stable angina (N=11). As a control a group, healthy volunteers gave a small blood sample (N=21).

The patients with CHD each had up to eight plasma samples taken while they underwent an angioplasty procedure. Some samples were taken before the stent was inserted (pre) and some after the stent was in place and the blood flow was restored (post). The sites the plasma was taken was from the femoral artery (FA), the aorta (AO pre and post), the coronary sinus (CS pre and post), the coronary artery (CA pre and post), and from the forearm vein 24 hours after the operation (24hr). Some patients were not able to give a sample from every site due to a low blood volume caused by the plaque blockage. The healthy volunteers gave just one sample which was taken from the forearm vein. Each plasma sample was analysed for neopterin and 7,8-dihydroneopterin, with all assays carried out in duplicate.

3.2.1 Plasma Neopterin Levels between Sample Sites.

Across the eight sample sites the plasma was obtained their was no significant difference in the concentration of neopterin or 7,8-dihydroneopterin (Figure 3.2.1and Figure 3.2.2). Prior to this study it was thought that sites around the heart, closest to the plaque, may contain more neopterin than away from the plaque such as the femoral artery site. Within this sample size of 35 patients not one showed a significant difference between the femoral artery and the other sites, between the pre and post stenting sites, or even 24 hours after the operation.

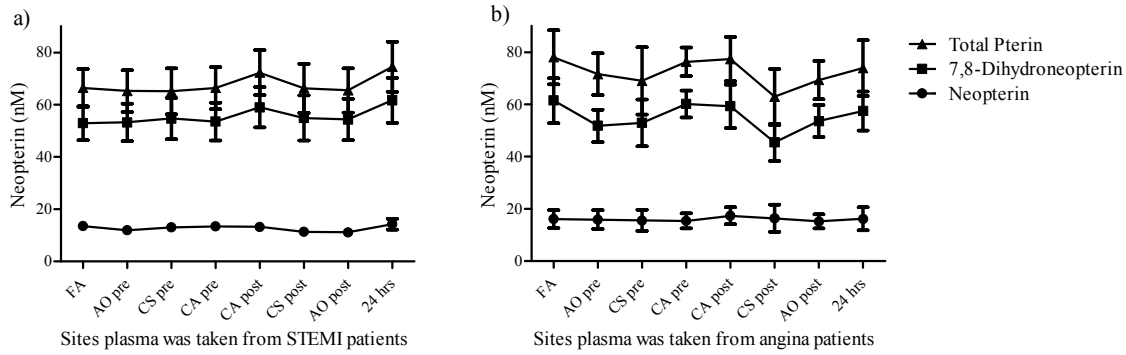

Figure 3.2.1 Concentrations of Neopterin, 7,8-Dihydroneoptein and Total Pterin in Patients with STEMI or Chronic Stable Angina
Plasma was taken from eight different sites, femoral artery (FA), Aorta (AO pre and post stent), coronary sinus (CS pre and post stent), coronary artery (CA pre and post stent) and 24 hours after operation (24hrs). Graph (a) shows the concentrations from patients undergoing an acute ST-elevation myocardial infarction (STEMI) (N=24), and graph (b) shows the concentrations from patients with chronic stable angina (N=11). Points represent the mean ± SEM.

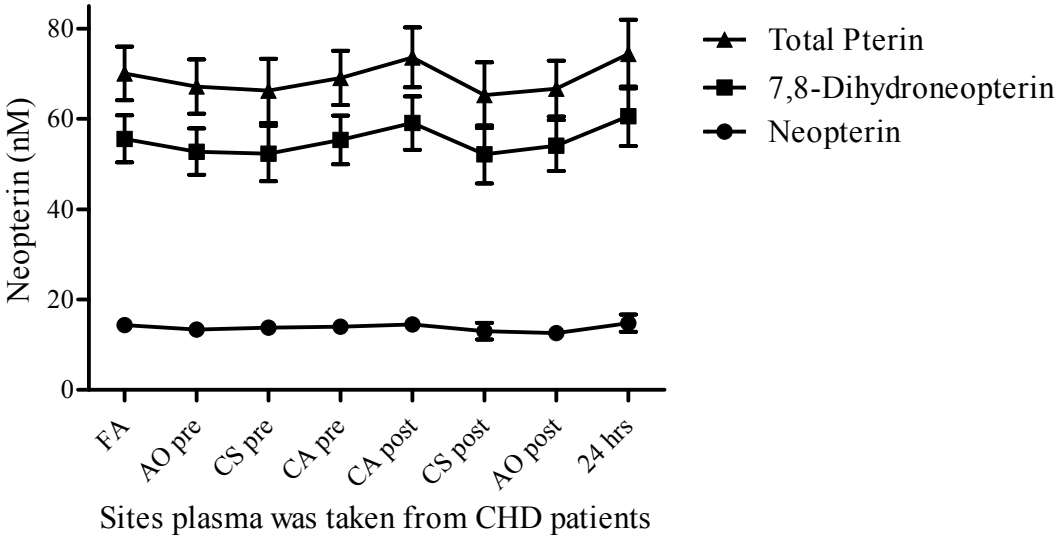

Figure 3.2.2 Concentrations of Neopterin, 7,8-Dihydroneoptein and Total Pterin in CHD Patients
Plasma data from cardio heart disease (CHD) patients, a combination of both groups STEMI and chronic angina patients, was analysed using the neopterin assay on the HPLC. The plasma was taken from eight different sites, femoral artery (FA), Aorta (AO pre and post stent), coronary sinus (CS pre and post stent), coronary artery (CA pre and post stent) and 24 hours after operation (24hrs). Points represent the mean ± SEM.

Figure 3.2.1 shows the separate data of both groups, while figure 3.2.2 contains the combined data of the CHD patients. Statistical analysis was carried out on both the STEMI group and the chronic angina group. There was no significant difference between

the two groups and therefore in the subsequent figures all data was combined into a general CHD group.

The average concentration of neopterin across all sample sites was 14.38 nM, the average concentration of 7,8-dihydroneopterin was 55.59 nM (Figure 3.2.2). This means that there was almost four times more 7,8-dihydroneopterin than neopterin in the plasma of cardio heart disease patients.

3.2.2 Coronary Heart Disease and Healthy Neopterin Concentrations

The data obtained from the femoral artery of the CHD patients was compared to the data from the healthy volunteers. The femoral artery was chosen as it was the only site that every patient gave a plasma sample from. The average neopterin concentration from the healthy volunteers was 6.83 ± 1.88 nM while the CHD patients average neopterin concentration was significantly higher ($P<0.01$) at 14.38 ± 1.53 nM. (Figure 3.2.3a) The difference between the 7,8-dihydroneopterin average concentrations were also highly significant ($P<0.001$) with the average healthy concentration at 17.78 ± 1.66 nM and the average CHD 7,8-dihydroneopterin concentration at 55.59 ± 5.19 nM (Figure 3.2.3b). Combined together the neopterin and 7,8-dihydroneopterin gave the total pterin concentration, this also showed that patients with CHD contained significantly more total pterin in their blood stream than the healthy volunteers (Figure 3.2.3c).

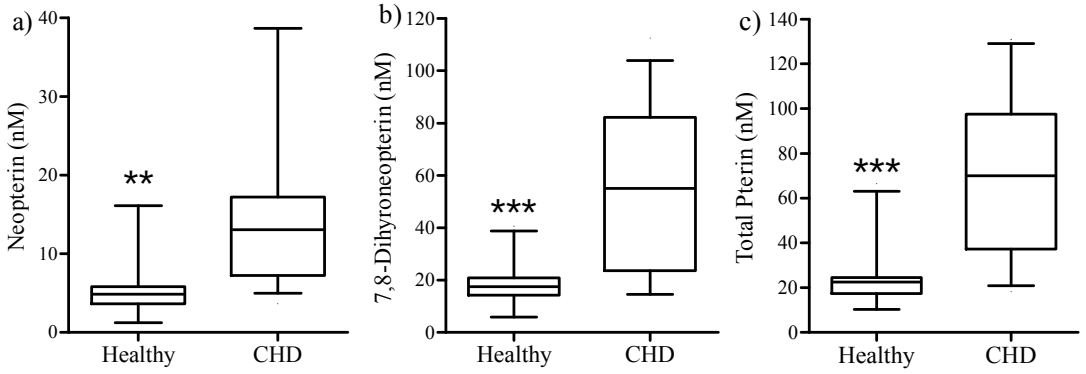

Figure 3.2.3 Comparison of Neopterin Levels in CHD Patients and Healthy Volunteers

For healthy volunteers (N=22), and CHD patients (N=34) plasma was analysed for neopterin (a), 7,8-dihydroneopterin (b) and total pterin (c). Data is presented as box plots with median lines and whiskers representing the 5th and 95th percentiles. Statistical significances are represented by ($P<0.05$) *, ($P<0.01$) **, ($P<0.001$)***.

3.2.3 Time from Onset of Chest Pain in STEMI Group and Neopterin Levels

The acute ST-elevation myocardial infarction (STEMI) patient's plasma samples were divided into two sets. They were divided by the time of the onset of the chest pain to the first blood sampling at the femoral artery, as either less than 180 minutes (N=11) or greater than 180 minutes (N=12). The samples taken less than 180 minutes from the onset of pain tended to have lower levels of neopterin (P=0.33), 7,8-dihydroneopterin (P=0.091) and total pterin (P=0.100) than the samples taken after 180 minutes. Although none of the markers showed a significant difference between the two time groups, 7,8-dihydroneopterin had the greatest separation between the averages. The average 7,8-dihydroneopterin concentration for patients who experienced less than 180 minutes of pain was 43.040 nM, while the average concentration for patients that had greater than 180 minutes of pain was 64.837 nM a 33% increase. This indicates that there is a possibility that the longer a myocardial infarction remains untreated, the greater the level of inflammation occurring. This could lead to a greater amount of 7,8-dihydroneopterin being produced.

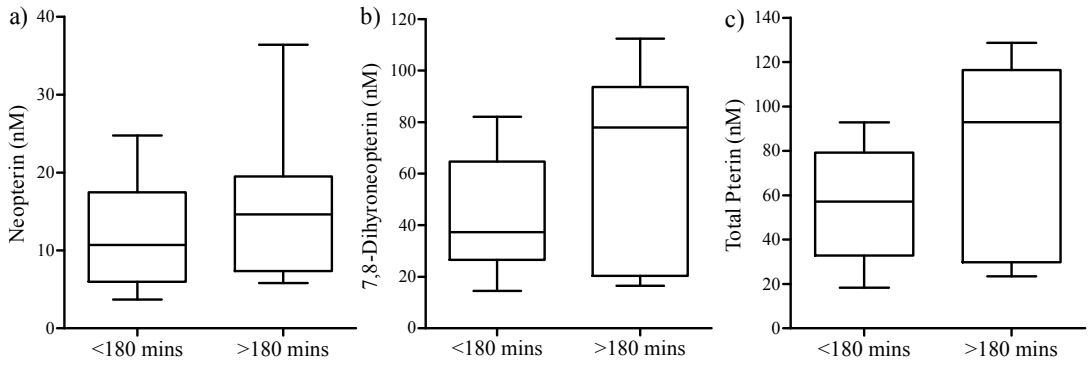

Figure 3.2.4 Comparison of Plasma in Patients with Acute STEMI Divided on Time of Onset of Pain.
For under 180 minutes (N=11), and for over 180 minutes (N=12) plasma was analysed for neopterin (a), 7,8-dihydroneopterin (b) and total pterin (c). All samples were taken from the femoral artery of the STEMI group of patients. Data is presented as box plots with median lines and whiskers representing the 5th and 95th percentiles. Statistical significances are represented by (P<0.05) *, (P<0.01) **, (P<0.001)***.

3.2.4 Comparison between Neopterin and Age

Other illnesses and diseases can often increase inflammation throughout the body and in turn increase the concentrations of neopterin and 7,8-dihydroneopterin in the plasma. Some of these illnesses and diseases include, diabetes, cancers and arthritis (Hamerlinck, 1999; Hoffmann et al., 2003), and they are often associated with increasing age.

The CHD patients in this study varied in age from 36 to 82 years old, and gave an even division of under 65's (N=17) and over 65's (N=18). In this group of patients there was very little difference in the neopterin and 7,8-dihydroneopterin concentrations between the under and over 65's. This suggests that the over 65 group had no serious underlying medical conditions, other than coronary heart disease, that increased their inflammation levels above the under 65 group.

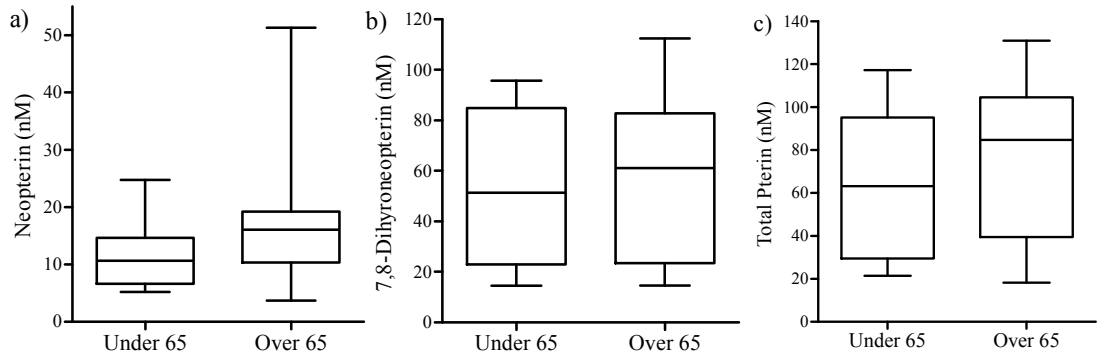

Figure 3.2.5 Comparison of Plasma from CHD Patients Dependent on Age.
The plasma was divided by age as under 65 (N=17) and over 65 (N=18), and it was then analysed for neopterin (a), 7,8-dihydroneopterin (b) and total pterin (c). All samples were taken from the femoral artery of the STEMI group of patients. Data is presented as box plots with median lines and whiskers representing the 5^{th} and 95^{th} percentiles. Statistical significances are represented by ($P<0.05$) *, ($P<0.01$) **, ($P<0.001$)***.

Discussion

A total of 19 advanced atherosclerotic plaques have been fully analysed by our laboratory, and each one has had its own unique biochemical composition. During this research it has become apparent that no two plaques develop in the same manner with the oxidant and antioxidant markers occurring in different concentrations not only between individual plaques but also between different sections within the same plaque. This variation can be seen in plaques C and E where there is over a 7 fold difference in the total 7-ketocholesterol content between the two plaques, with concentrations of 63.84 ± 4.96 and 454.84 ± 48.64 nmole/g of plaque respectively (Table III). The 7,8-dihydroneopterin content in plaque D shows how greatly markers can vary throughout plaque sections, 7,8-NP is only present in two sections of plaque D but in section 5 its concentration is highly elevated at 753.9 ± 68.5 pmole/g of plaque which is 26 times more than the amount of neopterin present in the same section (Figure 3.1.11). This variation in marker localisation and concentration levels indicates that plaque growth may occur under different mechanisms between separate plaques or between different sections of the same plaque.

Individual plaque correlations for plaques A-E showed a large amount of both positive and negative correlations between many of the markers (Table 3.1.7). Yet when the correlations were examined on the combined data a lesser number of correlations became significant and some appeared that had not been present in the individual correlations (Table 3.1.9).

While trends in the markers across the individual plaques appeared varied, splitting the marker concentrations into the three zones clearly showed the majority of the oxidation markers to be at their lowest concentrations in the post-bifurcation zone while this is where the antioxidant α-tocopherol was at its highest concentration (Figure 3.1.18 and Figure 3.1.19). These trends indicate that despite all the differences in the individual plaques there are general trends which could apply to most plaques.

Neopterin and 7,8-dihydroneopterin were both found to be significantly elevated in the plasma of patients with chronic stable angina and undergoing a myocardial infarction

compared to the healthy controls. 7,8-Dihydroneopterin's levels were on average 4 times higher than neopterin levels in the coronary heart disease patients indicating that while neopterin is a good marker of inflammation and the severity of atherosclerosis, obviously the majority of 7,8-dihydroneopterin is not immediately converted to neopterin and therefore both markers need to be measured to obtain a correct value of the inflammation in plaques.

4.1 Comparison of the Biochemical Marker Concentrations with Published Literature

From the twelve biochemical markers measured in the atherosclerotic plaques (A-S), eight have been previously quantified in published literature (Brown et al., 1997; Carpenter et al., 1993; Fu et al., 1998; Iuliano et al., 2003; Jachec et al., 2003; Micheletta et al., 2004; Nishi et al., 2002; Upston et al., 2002). There is currently no literature on the protein carbonyls AAS and GGS being measured within atherosclerotic plaque tissue by other laboratories. Even though Adachi et al. (2007) and Sugioka et al. (2010) have both detected neopterin in atherosclerotic plaques through immunohistochemical staining they did not quantify the concentration of neopterin present. Therefore our laboratory is the first to quantify the levels of neopterin and 7,8-dihydroneopterin, as well as AAS and GGS in atherosclerotic plaques.

The average amount of total cholesterol within the plaques (A-S), was 48.41 ± 6.30 μmole/g of plaque, this is in agreement with Iuliano et al. (2003) and Micheletta et al. (2004) who both determined the cholesterol content of carotid plaques to be 40.6 ± 28.7 μmole/g tissue. The protein concentration in atherosclerotic plaques has been quantified at 1.49 ± 0.62 mg/ml of plaque homogenate by Upston et al. (2002). Although lower it is still comparable to the average protein concentration for plaque A-S of 2.23 ± 0.35 mg/ml of plaque homogenate.

α-Tocopherol has been quantified within plaque tissue by several research groups as both nmole/g of plaque tissue and as mmole/mol of cholesterol. The average α-tocopherol concentration for plaques A-S was 123.28 ± 20.41 nmole/g of plaque, which is similar with Carpenter et al. (1993), Iuliano et al. (2003) and Micheletta et al. (2004) who

reported α-tocopherol concentrations of 156 ± 118, 85.6 ± 62.1 and 71.3 ± 49.2 nmole/g plaque tissue respectively. The α-tocopherol level, expressed as α-tocopherol per mole of cholesterol, for plaques A-S was 2.73 ± 0.33 mmole/mol of cholesterol, was within the range of values given by Carpenter et al. (1993), Micheletta et al. (2004) and Upston et al. (2002) which was between 2.0 and 5.4 mmole/mol of cholesterol.

Nishi et al. (2002) reported a TBARS average concentration of 35.9 ± 3.1 nmole/g of plaque tissue which is higher than the average for the plaques A-S of 12.26 ± 3.75 nmole/g of plaque. TBARS concentration varied dramatically throughout the plaques A-S with the lowest concentration recorded at 3.46 nmole/g and the highest concentration at 76.31 nmole/g of plaque. This variation in TBARS concentration within atherosclerotic plaques was also detailed in Jachec et al. (2003) who reported a range of 0.23 to 37.36 μg/g of tissue. Unfortunately the concentrations of plaques A-S can not be directly compared to the values given in Jachec et al. (2003) due to the difference in the units of measure, but it can be concluded that the highly variable concentrations of TBARS between different plaques has been previously quantified. Within plaques A-S the average concentrations for DOPA and dityrosine were 14.42 ± 1.67 and 4.44 ± 0.89 nmole/g of plaque tissue respectively. These values agree with previous DOPA and dityrosine concentrations measured in carotid plaques of 14.26 ± 3.8 and 4.75 ± 5.17 nmole/g of plaque tissue respectively (Fu et al., 1998). As the 7-ketocholesterol assay was only introduced with plaque B just four plaques have the concentrations for free and total 7-ketocholesterol. Plaque C had the lowest overall concentration of 15.55 nmole/g of plaque for free and 63.84 ± 4.96 nmole/g of plaque for total. While plaque E had the highest concentration of 108.32 nmole/g of plaque for free and 454.84 ± 48.64 nmole/g of plaque for total. The averages for the four plaques for the free 7-ketocholesterol was 63.68 ± 19.34 nmole/g of plaque was higher than that published by Iuliano et al. (2003) and Micheletta et al. (2004) of 35.9 ± 21.5 and 39.7 ± 14.0 nmole/g of tissue respectively. However Jachec et al. (2003) showed atherosclerotic plaques to have a free 7-ketocholesterol concentration range of 14.8 to 577.5 ng/g of tissue. Even though this data can not be directly compared to the data from plaques B-E due to the difference in the units of measurement value (nmoles/g vs. ng/g) it does show that large variations in free 7-ketocholesterol concentrations within plaques has been previously documented. Brown et al. (1997) detailed in their paper the 7-ketocholesterol content in two carotid

plaques. They found the free 7-ketocholestrol level to be 17 ± 21 nmole/g of plaque, which is much lower than our average but similar to the amount found in plaque C. They found the concentration of total (free plus esterified) 7-ketocholesterol to be 117 ± 42 nmole/g of plaque, this agrees with data form plaques B-D. Within human atherosclerotic plaques 80-95% of the 7-ketocholesterol of esterified (Brown et al., 1997; Brown & Jessup, 2009), in plaques B-D 71% appeared to be esterified.

4.2 Correlations between the Markers across the Atherosclerotic Plaques

There was a high degree of variation in the marker concentrations and correlations between the individual plaques. This may possibly occur due to the differences in the patients from whom the plaques where taken, but also due to variations in the age or stage of growth each individual plaque was in. It is presumed that the chemical composition of each lesion changes continuously due to variations in the oxidant-antioxidant balance and the lipid content of the diet. Despite these differences in the plaques, significant correlations were seen between many pairs of markers. These correlations may provide insight into the interactions and influences the markers have on each other.

α-Tocopherol

α-Tocopherol correlated positively with cholesterol in all five of the plaques and in the combined plaque data (Table 3.1.6 and Table 3.1.9). This was the strongest correlation between two markers to be seen in all the plaque data. Both α-tocopherol and cholesterol are brought into the plaque by LDL. α-Tocopherol is the most prevalent antioxidant in LDL with 5-9 in each LDL particle (Pryor., 2000). While LDL is the major carrier of cholesterol in macrophage foam cells with atherosclerotic plaques (Brown et al., 1997; Kruth et al., 2002). α-Tocopherol also had a very strong positive correlation ($p<0.001$) with protein in the combined data, this was also seen in by Crone (2008) and Flavall (2008) in their combined plaque data. Although there were no correlations seen in any of the individual plaques A-E, only plaque N (Crone., 2008) has shown a positive correlation between these two markers. It is unclear what the connection between α-tocopherol and protein may be.

Protein Oxidation

In the combined plaque data DOPA, the protein carbonyls AAS and GGS all correlated with protein. This could be expected as protein is the major substrate for their oxidation. The correlations in the individual plaques between these protein oxidation products, including dityrosine, and protein was not seen in all plaques possibly due to their specificity. DOPA and dityrosine are formed by oxidation of tyrosine, while AAS and GGS protein carbonyls are formed by the metal ion-catalyzed oxidation of proline, arginine and lysine residues. Because AAS and GGS are formed by the same process it is not surprising that they both positively correlate with each other, in the combined plaque data as well as in four of the five individual plaques. DOPA and dityrosine also correlated together in the combined data. This has been previously observed by both Crone (2008) and Flavall (2008) indicating that overall these two markers appear to be formed by the same process within the artery wall. Across all plaques the levels of DOPA were consistently higher than those of dityrosine, although not every plaque or section contained detectable levels of dityrosine. This difference in the levels of DOPA and dityrosine has also been previously documented in Fu et al. (1998) and a reason for this may be that dityrosine is formed from the dimerization of two tyrosine phenoxyl radicals where the levels are critically dependant on the radical flux and do not increase in a linear manner with increasing radical concentrations (Fu et al., 1998). Therefore the differing levels of dityrosine per parent tyrosine might indicate that the levels of radical flux differ along and between atherosclerotic plaques.

Lipid Peroxidation

Total 7-ketocholesterol positively correlated with cholesterol in the combined plaque data but only plaque E showed a correlation between the two for the individual plaques. 7-Ketocholesterol is formed when a free radical directly attacks at cholesterols seventh carbon. Esterified 7-Ketocholesterol formed from either esterified cholesterol or esterified after oxidation makes up 80-95% of the total 7-KC found in plaques (Brown & Jessup, 2009) which is most likely why in the combined data only the total 7-ketocholesterol correlated with the cholesterol and not the free. As they have the same method of formation, the free and total 7-ketocholesterol were expected to correlate positively with each other. While only free 7-ketocholesterol correlated to TBARS in the combined data, total 7-ketocholesterol also show positive correlations with TBARS in

the individual plaque data. This correlation may exist as both are lipid oxidation products, and Nishi et al. (2002) showed there was a strong correlation between lipid peroxidation and the total lipid content within atherosclerotic plaques. TBARS had a positive correlation with neopterin in the combined data as well as in two of the individual plaques. This correlation may occur as neopterin and TBARS are oxidation products and are likely to both be formed at sites of oxidative stress.

The lipid peroxidation products, free 7-ketocholesterol and TBARS, correlated with AAS and GGS protein carbonyls in the combined plaque data. The connection and therefore the correlations between these lipid peroxidation products and protein oxidation products may have occurred as a result of the formation and decomposition of lipid peroxides in the presence of redox-active iron to yield aldhydes (Li et al., 2006), or when lipid peroxidation takes place the generation of reactive oxygen species in turn oxidises nearby proteins (Berlett & Stadtman, 1997)

4.3 Effect of Zoning and Shear Stress on Marker Concentrations in Atherosclerotic Plaques.

It has been well established that atherosclerotic plaques often occur in specific areas in the arteries, such as branch points and areas of major curvature, where disturbed blood flow causes endothelial shear stress (ESS) (Chatzizisis et al., 2007, Gimborne, 1999). The level of shear stress differs across the length of the plaque and it is known to disrupt the function of endothelial cells. Since the endothelial cells form the barrier between the intima and the circulating blood, shear stress possesses the ability to cause LDL to accumulate and therefore it can affect the concentrations of cholesterol and α-tocopherol across the plaque (Chapman, 2007). The cholesterol content increased in the bifurcation and post-bifurcation zones, while α-tocopherol was greatly increased in the post-bifurcation zone (Figure3.1.18). These trends may be explained by the occurrence of low or oscillatory ESS which has been reported to increase the accumulation of lipids (Chatzizisis et al., 2007).

The neopterin concentration was at the highest levels in the pre-bifurcation zone, this region of the plaque this thought of as the leading edge of the plaque. This is the newest area of plaque growth, it has the greater number of activated macrophages with little to no gruel. The gruel from carotid plaques has been shown to highly toxic to human

macrophages (Li et al., 2006) therefore the macrophages present within the gruel, which is predominantly found in the bifurcation and post-bifurcation zones, are most likely to be dead. This could explain why the level of neopterin is so low in the bifurcation zone. While all five plaques (A-E) where analysed for the presence of 7,8-dihydroneopterin it was only detected in a few sections in three of the plaques. It is possible the highly oxidative environment within the plaques oxidised the 7,8-dihydroneoptrin to neopterin. Therefore there is not enough data at this stage to give a picture of where in the plaque it may localise. In plaque C, 7,8-dihydroneopterin was present in relatively equal amounts throughout the length of the plaques four sections, plaque C did not have any pre-bifurcation sections (Figure 3.1.8). In plaque D, 7,8-dihydroneopterin was found in the first section of the post-bifurcation zone (Figure 3.1.11) and in plaque E one section from the pre-bifurcation and one section from the bifurcation zones contained 7,8-dihydroneopterin (Figure 3.1.14). This small amount of data from 7,8-dihydroneopterin seems to suggest that it is not elevated in the pre-bifurcation zone with a reduced level in the bifurcation zone as neopterin is, but a larger data set is needed to show a definite trend.

All the plaques analysed were in an advanced stage of atherosclerosis with between 60-95% stenosis. This stenosis generally occurred in either the bifurcation or post-bifurcation zones, where the artery is narrowed and the amount of gruel and/or calcium is increased. Therefore blood flood in the pre-bifurcation zone would slow down and start to re-circulate around as it is unable to flow normally through the stenosed region. The presence of stenosis and the re-circulation of blood may result in a low and oscillating shear stress within the pre-bifurcation zone (Deplano & Siouffi, 1999). Low ESS promotes the production of reactive oxygen species (ROS) in the intima eventually leading to the oxidation of LDL, it also down regulates the intracellular ROS scavengers (Chatzizisis et al., 2007). This could explain why the lipid oxidation makers, TBARS and 7-ketocholesterol, are elevated in the pre-bifurcation zone. The increase of ROS, and the decrease of ROS scavengers and α-tocopherol could lead to the significantly high levels of the protein carbonyls AAS and GGS in the pre-bifurcation zone. Also the high levels of neopterin in the pre-bifurcation zone may influence the concentration of the oxidation markers as neopterin is believed promote the oxidative potential of reactive oxygen species leading of oxidative stress as well (Adachi et al., 2007; Schroecksnadel et al.,

2006). The level of DOPA is only slightly higher in the pre-bifurcation zone compared to the other two zones, and the dityrosine levels show an opposite trend to all the other oxidation markers with its lowest concentration in the pre-bifurcation zone and a significant increase in the bifurcation and post-bifurcation zones. The reason for dityrosine's trend is unclear, it may be due to the specificity in which dityrosine is formed. Or possibly because dityrosine is not always found in all sections of the plaque, many plaques have sections with undetectable levels of dityrosine and this may alter the zoning results.

4.4 Concentrations of Neopterin and 7,8-Dihydroneopterin in the Plasma of Coronary Heart Disease Patients.

The elevated concentration of neopterin in the plasma of patients suffering from both acute (myocardial infarctions) and chronic (stable and unstable angina) coronary heart diseases is well documented (Avanzas et al., 2005; Djordjevic et al., 2008; García-González et al., 2008; Garcia-Moll et al., 2000; Murr et al., 2009; Sahin et al., 2008; Schumacher et al., 1997; Sugioka et al., 2010; Tatzber et al., 1991; Videm et al., 2007). In this study the levels of neopterin and 7,8-dihydroneopterin in patients with chronic stable angina and those who had just suffered an acute ST-elevation myocardial infarction (STEMI) were significantly elevated compared to a control group of healthy volunteers. There was no significant variation in the concentrations of neopterin or 7,8-dihydroneopterin between the two patient groups, therefore the amount of inflammation appears to be the same regardless of whether the disease was acute or chronic.

Plasma neopterin levels in patients with coronary artery disease have been well studied with many publications giving the neopterin levels for these types of patients. But there is no published literature showing the levels of both neopterin and 7,8-dihydroneopterin together in patients with coronary heart disease or in healthy control subjects. In our study the average neopterin concentration in the plasma of patients with coronary heart disease (CHD) was 14.38 ± 1.53 nM, this is in agreement with the previously published average neopterin values for CHD patients of 17.9 nM (Djordjevic et al., 2008), 10.2 nM (García-González et al., 2008), 18.5 nM (Sahin et al., 2008), 13.7 nM (Schumacher et al., 1997), 15.5 nM (Tatzber et al., 1991) and 17.4 nM (Videm et al., 2007). The average neopterin concentration for healthy volunteers in our study was 6.83 ± 1.88 nM, which is

also within the range of neopterin values given for the healthy volunteers published as 10.9 nM (Djordjevic et al., 2008), 6.98 nM (García-González et al., 2008), 8.7 nM (Sahin et al., 2008), 6.8 nM (Schumacher et al., 1997) and 5.0 nM (Tatzber et al., 1991).

The levels of 7,8-dihydroneopterin in patients with coronary heart disease (CHD) were previously un-quantified, although it is thought that the ratio of neopterin to 7,8-dihydroneopterin in venous blood is 1:2 and in arterial blood is 1:3 (Hoffmann et al., 2003; Murr et al., 2002). The amount of 7,8-dihydroneopterin was 3.87 times greater than the neopterin in the CHD patient where the blood was taken from the arteries, and 2.6 times greater than neopterin in the control group where the blood was taken from the forearm vein. So while the concentrations of 7,8-dihydroneopterin were significantly elevated in the CHD patients compared to the controls, the levels of both groups are still within the expected ratios to neopterin to 7,8-dihydroneopterin. This indicates that even though significantly more inflammation is occurring in the CHD patients, the 7,8-dihydroneopterin that is entering the blood stream away from its site of production in the plaque may not be becoming oxidised to neopterin by HOCl. In the plaque samples A-E, 7,8-dihydroneopterin was only found in three of these plaques and even then it was not present in all sections. Plaque C had no detectable levels of neopterin but 7,8-dihydroneopterin was found in all sections, and in section 5 of plaque D the 7,8-dihydroneopterin concentration was 26 times greater than the neopterin concentration. This 7,8-dihydroneopterin that is found in the plaque samples maybe newly synthesized from active macrophages and it might eventually become fully oxidised to neopterin. This could explain the extremely high levels of neopterin found within the atherosclerotic plaques, with an average concentration of 1202 nM in plaques A-S compared to the 14.38 nM found in the plasma of CHD patients.

Patients suffering an acute STEMI were divided into two groups depending on whether their first blood sample was taken either less than 180 minutes or greater than 180 minutes from the onset of pain. There was no significant differences in the neopterin or 7,8-dihydroneopterin concentrations between the two groups. Although 7,8-dihydroneopterin had the greatest separation between the averages possibly indicating that the longer a myocardial infarction remains untreated the more inflammation that could occur. When the CHD patients were divided by age, there was no significant difference in the concentration of neopterin or 7,8-dihydroneopterin between the over 65

and under 65 age groups. Neopterin levels are known to increase with age, most likely due to age related inflammatory diseases (Murr et al., 2002), but that was not seen in this group of patients indicating the older patients had no serious underlying medical conditions, other than coronary heart disease, that increased their inflammation levels above the younger patients

4.5 Summary

The major finding of this study is that oxidant, anti-oxidant and inflammatory markers vary greatly along the length of individual plaques and between whole plaques. While no two plaques contained the exact same biochemical composition general trends were observed with the combined plaque data. Correlations between markers indicated that relationships do occur within the plaque. Zoning within the plaque showed that endothelial shear stress caused by blood flow results in a separation between the oxidants in the pre-bifurcation zone and α-tocopherol in the post-bifurcation zone. The collection of plaque data is still ongoing, with the addition of more data stronger correlations and zoning trends may be revealed. This may help to understand the marker composition within advanced atherosclerotic plaques better, although variations among plaques will always exist due to the differences in biochemistry from the patients in which they were taken.

The plasma neopterin and 7,8-dihydroneopterin concentrations were significantly elevated in patients with both acute and chronic coronary heart disease compared to healthy control volunteers. While neopterin was already well known to be elevated, this was the first study that showed 7,8-dihydroneopterin to also be significantly elevated.

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Appendix

Table I. Extended Version of Table 3.1.6 Correlation of Inflammatory and Oxidative Markers within Plaques A-E

Significant correlations were determined based on the average of each section a (n=3) within plaques A-E. N is the number of sections within each plaque. Combined plaque analysis was performed on plaques A-N, P-R, where N is the collated number of sections of the plaques. For neopterin correlations plaques F-I were excluded. For AAS and GGS protein carbonyls correlations only data from plaques from A-E was analysed, and for 7-ketocholesterol correlations only data from plaques B-E was analysed. Protein was expressed in mg/g plaque, cholesterol μmole/g plaque and neopterin, α-tocopherol, TBARS, DOPA, Dityrosine, AAS and GGS protein carbonyls and 7-ketocholesterol as nmole/g plaque. Statistical significance represented by P<0.05 * P<0.01 ** P<0.001 ***

Markers	r(Pearson)	P value		N
Plaque A				
Cholesterol vs. Protein	-0.4636	0.0152	*	9
Cholesterol vs. Neopterin	-0.7070	0.0004	***	9
Cholesterol vs. α-tocopherol	0.8489	0.0001	***	9
Neopterin vs. α-tocopherol	-0.4256	0.0273	*	9
Neopterin vs. DOPA	-0.4078	0.0352	*	9
Dityrosine vs. Protein	-0.4213	0.0294	*	9
Dityrosine vs. Cholesterol	0.7177	0.0005	***	9
Dityrosine vs. Neopterin	-0.5714	0.0022	**	9
Dityrosine vs. α-tocopherol	0.4999	0.0083	**	9
AAS protein carbonyl vs. Protein	0.5005	0.0081	**	9
AAS protein carbonyl vs. Cholesterol	-0.5301	0.0043	**	9
AAS protein carbonyl vs. Neopterin	0.7093	0.0004	***	9
AAS protein carbonyl vs. α-tocopherol	-0.4328	0.0241	*	9
GGS protein carbonyl vs. Cholesterol	-0.5889	0.0012	**	9
GGS protein carbonyl vs. Neopterin	0.7249	0.0003	***	9
GGS protein carbonyl vs. α-tocopherol	-0.4948	0.0091	**	9
GGS protein carbonyl vs. TBARS	0.4075	0.0352	*	9
GGS protein carbonyl vs. DOPA	-0.4478	0.0191	*	9
GGS protein carbonyl vs. Dityrosine	-0.4173	0.0302	*	9

Table I Continued. Extended Version of Table 3.1.6 Correlation of Inflammatory and Oxidative Markers within Plaques A-E

Markers	r(Pearson)	P value		N
GGS protein carbonyl vs. AAS	0.9700	0.0001	***	9
Plaque B				
Cholesterol vs. α-tocopherol	0.8741	0.0002	***	6
Protein vs. TBARS	0.8684	0.0001	***	6
Protein vs. DOPA	-0.5582	0.0162	*	6
Protein vs. AAS protein carbonyl	0.6293	0.0053	**	6
Protein vs., Free 7-ketocholesterol	0.4913	0.0381	*	6
α-tocopherol vs. GGS protein carbonyl	0.5345	0.0223	*	6
TBARS vs. DOPA	-0.7600	0.0002	***	6
TBARS vs. AAS protein carbonyl	0.7770	0.0001	***	6
AAS protein carbonyl vs. DOPA	-0.7593	0.0003	***	6
AAS protein carbonyl vs. GGS	0.6653	0.0032	***	6
Plaque C				
Cholesterol vs. Protein	0.7507	0.0052	**	4
Cholesterol vs. α-tocopherol	0.7916	0.0023	**	4
Cholesterol vs. AAS protein carbonyl	0.7097	0.0101	*	4
AAS protein carbonyl vs. α-tocopherol	0.8009	0.0023	**	4
AAS protein carbonyl vs. GGS	0.6115	0.0354	*	4
DOPA vs. Total 7-ketocholesterol	0.7426	0.0061	**	4
Plaque D				
Protein vs. Neopterin	0.9386	0.0001	***	6
Protein vs. TBARS	0.9839	0.0002	***	6
Protein vs. DOPA	0.6744	0.0022	**	6
Protein vs. Dityrosine	-0.7259	0.0013	**	6
Cholesterol vs. α-tocopherol	0.6351	0.0051	**	6
Neopterin vs. TBARS	0.9672	0.0002	***	6
Neopterin vs. DOPA	0.4837	0.0423	*	6
Neopterin vs. Dityrosine	-0.7356	0.0011	**	6

Table I Continued. Extended Version of Table 3.1.6 Correlation of Inflammatory and Oxidative Markers within Plaques A-E

Markers	r(Pearson)	P value		N
TBARS vs. DOPA	0.6625	0.0032	**	6
TBARS vs. Dityrosine	-0.7439	0.0001	***	6
AAS protein carbonyl vs. GGS	0.9772	0.0003	***	6
Free 7-ketocholesterol vs. Protein	0.9758	0.0001	***	6
Free 7-ketocholesterol vs. Neopterin	0.9625	0.0001	***	6
Free 7-ketocholesterol vs. TBARS	0.9839	0.0003	***	6
Free 7-ketocholesterol vs. DOPA	0.5806	0.0123	*	6
Free 7-ketocholesterol vs. Dityrosine	-0.7389	0.0002	***	6
Total 7-ketocholesterol vs. Protein	0.9531	0.0001	***	6
Total 7-ketocholesterol vs. Neopterin	0.9411	0.0001	***	6
Total 7-ketocholesterol vs. TBARS	0.9708	0.0002	***	6
Total 7-ketocholesterol vs. DOPA	0.5968	0.0093	**	6
Total 7-ketocholesterol vs. Dityrosine	-0.7583	0.0004	***	6
Total 7-ketocholesterol vs. Free 7-kc	0.9609	0.0002	***	6
Plaque E				
Neopterin vs. Protein	0.5167	0.0283	*	6
Neopterin vs. Cholesterol	0.4985	0.0352	*	6
Neopterin vs. Total pterin	0.4872	0.0403	*	6
Neopterin vs. TBARS	0.6204	0.0061	**	6
Total pterin vs. TBARS	0.5432	0.0203	*	6
TBARS vs. Protein	0.5525	0.0174	*	6
TBARS vs. DOPA	0.7288	0.0013	**	6
Cholesterol vs. α-tocopherol	0.7500	0.0002	***	6
Dityrosine vs. Protein	0.5258	0.0251	*	6
Dityrosine vs. TBARS	0.5237	0.0262	*	6
Dityrosine vs. DOPA	0.6020	0.0083	**	6
Dityrosine vs. GGS protein carbonyl	0.5329	0.0231	*	6
GGS protein carbonyl vs. Protein	0.5358	0.0222	*	6
Free 7-ketocholesterol vs. Protein	0.6914	0.0013	**	6

Table I Continued. Extended Version of Table 3.1.6 Correlation of Inflammatory and Oxidative Markers within Plaques A-E

Markers	r(Pearson)	P value		N
Free 7-ketocholesterol vs. Cholesterol	0.4758	0.0461	*	6
Free 7-ketocholesterol vs. DOPA	0.4924	0.0382	*	6
Total 7-ketocholesterol vs. Protein	0.6764	0.0023	**	6
Total 7-ketocholesterol vs. Cholesterol	0.5740	0.0131	*	6
Total 7-ketocholesterol vs. Neopterin	0.6855	0.0012	**	6
Total 7-ketocholesterol vs. Total pterin	0.5826	0.0113	*	6
Total 7-ketocholesterol vs. TBARS	0.5451	0.0191	*	6
Total 7-ketocholesterol vs. GGS	0.5593	0.0162	*	6
Total 7-ketocholesterol vs. Free 7-kc	0.7257	0.0011	**	6

Table II. Summary of the Average Overall Concentrations of Protein, Cholesterol, Neopterin, Total Pterin, α-Tocopherol and TBARS in Plaques A-S.
Plaques A-E analysed in this study, F-G in Firth (2006), H-M in Flavall (2008) and N-S in Crone (2008). Data represents mean ± SEM of the section averages. ND = Not detected.

Plaque (Lab N^{o})	Protein	Cholesterol	Neopterin & Total Pterin	α-Tocopherol	TBARS
	mg/g plaque	µmole/g plaque	nmole/g plaque nmole/g plaque	nmole/g plaque	nmole/g plaque
A (17)	93.68 ± 3.80	58.99 ± 6.85	1.77 ± 0.13	139.41 ± 13.45	4.75 ± 0.17
B (19)	136.34 ± 7.09	32.10 ± 2.56	ND ND	86.52 ± 7.78	4.73 ± 0.45
C (21)	119.93 ± 15.09	34.93 ± 8.49	ND 0.01 ± 0.00	157.84 ± 23.46	8.00 ± 0.03
D (23)	151.1 ± 21.98	52.90 ± 4.39	0.06 ± 0.01 0.20 ± 0.06	235.08 ± 26.21	10.99 ± 2.60
E (24)	86.20 ± 3.63	62.87 ± 3.53	0.05 ± 0.01 0.13 ± 0.03	151.67 ± 10.14	3.88 ± 0.25
F (3)	77.62 ± 4.49	37.15 ± 5.01	0.21 ± 0.01*	32.33 ± 4.96	4.59 ± 0.70
G (4)	66.71 ± 4.02	84.75 ± 12.10	0.16 ± 0.01*	44.49 ± 2.84	3.39 ± 0.41
H (5)	70.30 ± 6.19	75.82 ± 8.21	0.09 ± 0.00*	57.04 ± 6.57	7.48 ± 0.77
I (6)	92.04 ± 5.13	80.89 ± 6.38	0.21 ± 0.01*	37.94 ± 5.72	7.43 ± 1.13
J (7)	64.26 ± 2.20	57.95 ± 4.47	1.59 ± 0.11	127.09 ± 6.84	4.53 ± 0.85
K (8)	92.14 ± 3.22	47.42 ± 3.28	1.79 ± 0.15	213.09 ± 6.84	7.13 ± 1.15
L (9)	94.78 ± 13.10	25.81 ± 2.20	1.26 ± 0.18	60.00 ± 5.54	14.28 ± 1.59
M (10)	140.33 ± 5.45	29.31 ± 3.95	0.92 ± 0.06	152.27 ± 10.42	15. 49 ± 1.61
N (11)	69.23 ± 7.06	7.85 ± 0.38	2.34 ± 0.41	21.84 ± 1.66	21.31 ± 4.41
O (12)	45.16 ± 6.02	6.68 ± 0.69	1.57 ± 0.16	9.64 ± 1.79	76.32 ± 16.49
P (14)	104.29 ± 6.14	43.39 ± 4.94	2.02 ± 0.15	161.70 ± 16.27	12.31 ± 1.24
Q (15)	120.47 ± 4.10	64.21 ± 2.34	1.93 ± 0.15	95.40 ± 5.25	12.16 ± 0.30
R (16)	186.59 ± 22.78	44.86 ± 4.46	2.00 ± 0.29	200.56 ± 25.30	4.20 ± 0.29
S (17)	91.76 ± 4.04	92.61 ± 0.41	0.72 ± 0.01	360.20 ± 24.74	9.84 ± 0.68
Overall Average	**100.23 ± 8.01**	**51.52 ± 5.64**	**1.20 ± 0.22** **0.12 ± 0.03**	**123.28 ± 20.41**	**12.26 ± 3.72**

Table III. Summary of the Average Overall Concentrations of DOPA, Dityrosine, AAS and GGS Protein Carbonyls, General Protein Carbonyls, Free 7-Ketocholesterol and Total 7-Ketocholesterol in Plaques A-S.
Plaques A-E analysed in this study, F-G in Firth (2006), H-M in Flavall (2008) and N-S in Crone (2008). The AAS and GGS protein carbonyl method replaced the old protein carbonyl method in plaques A-E. The 7-ketocholesterol assay was only introduced from plaque B-E. Data represents mean ± SEM of the section averages. ND = Not detected.

Plaque	DOPA	Dityrosine	AAS GGS	Protein Carbonyls	Free 7-KC Total 7-KC
	nmole/g plaque	nmole/g plaque	nmole/g plaque nmole/g plaque	nmole/g plaque	nmole/g plaque nmole/g plaque
A (17)	13.15 ± 0.75	2.19 ± 0.70	59.29 ± 7.02 25.69 ± 5.24		
B (19)	7.99 ± 1.49	7.77 ± 1.59	109.25 ± 9.48 15.39 ± 1.32		56.03 ± 9.60 190.03 ± 26.63
C (21)	24.69 ± 1.64	6.19 ± 0.76	28.77 ± 3.02 3.65 ± 0.37		15.55 ± 2.04 63.84 ± 4.96
D (23)	27.29 ± 1.60	2.70 ± 0.37	99.78 ± 14.24 42.45 ± 3.24		74.85 ± 22.48 157.53 ± 39.60
E (24)	8.86 ± 0.48	3.17 ± 0.32	36.87 ± 1.84 15.33 ± 0.81		108.32 ± 9.18 454.84 ± 48.64
F (3)	12.79 ± 0.66	0.50 ± 0.10		161.52 ± 25.88	
G (4)	11.58 ± 0.67	4.86 ± 1.21		357.32 ± 19.78	
H (5)	15.66 ± 0.71	0.59 ± 0.14		310.21 ± 32.37	
I (6)	29.13 ± 3.45	11.62 ± 2.92		140.86 ± 13.26	
J (7)	4.93 ± 0.61	0.91 ± 0.15		287.52 ± 25.25	
K (8)	15.50 ± 3.47	8.86 ± 1.54		445.04 ± 50.85	
L (9)	7.59 ± 1.96	ND		276.18 ± 46.51	
M (10)	10.80 ± 0.70	9.19 ± 0.89		389.90 ± 38.25	
N (11)	6.44 ± 0.76	0.05 ± 0.03		266.54 ± 62.89	
O (12)	7.01 ± 0/81	ND		159.63 ± 47.15	
P (14)	25.41 ± 2.04	9.11 ± 2.35		369.50 ± 29.29	
Q (15)	16.22 ± 1.08	9.86 ± 1.21		137.32 ± 30.96	
R (16)	13.86 ± 1.47	2.27 ± 0.61		434.20 ± 35.63	
S (17)	16.24 ± 1.63	3.94 ± 0.12		256.10 ± 56.94	
Overall Average	**14.41 ± 1.66**	**4.44 ± 0.89**	**66.79 ± 16.26** **20.50 ± 6.50**	**287.27 ± 28.93**	**63.69 ± 19.35** **216.56 ± 83.81**

Table IV. Neopterin, 7,8-Dihydroneopterin and Total Pterin Concentrations in STEMI Patients.

Concentrations neopterin and total pterin are in nM ± SEM of duplicates. Concentrations of 7,8-dihydroneopterin are in nM, obtained by subtracting the neopterin concentration from the total pterin concentration. FA – femoral artery, AO pre – aorta pre stent, CS pre – coronary sinus pre stent, CA pre – coronary artery pre stent, CA post – coronary artery post stent, CS post – coronary sinus post stent, AO post – aorta post stent, 24 hr – 24 hours after the operation.

	FA	AO pre	CS pre	CA pre	CA post	CS post	AO post	24 hr
RSE	23.36±0.74	22.70±0.63	20.21±1.14	21.97±0.84	19.78±0.02	17.76±2.94	21.71±1.30	14.08±0.10
	93.80	65.98	63.44	117.54	116.18	122.37	100.81	97.34
	117.8±13.2	88.69±7.64	83.66±3.12	139.51±4.1	135.96±4.2	140.13±1.5	122.5±18.9	111.42±0.3
AM	16.15±1.56	15.05±0.11	18.86±0.38	19.34±0.09	17.26±1.85		18.11±0.05	18.72±0.18
	112.49	93.37	108.81	104.27	89.09		81.71	101.69
	128.64±2.3	108.43±1.2	127.67±1.5	123.61±0.9	106.4±11.9		99.83±14.2	120.42±3.5
WW	15.25±1.11	14.46±0.48	16.10±0.18	16.08±0.25	15.57±0.06	16.98±1.06	16.03±0.08	17.77±0.09
	36.35	55.33	54.52	45.41	68.90	60.54	42.53	61.77
	51.60±5.83	69.79±3.94	70.62±2.40	61.50±9.36	84.48±0.07	77.53±0.70	58.56±0.17	79.54±0.15
PMU	17.45±0.06			15.27±0.36	13.70±0.16			
	46.61			44.95	52.62			
	64.07±3.25			60.22±5.25	66.32±0.53			
OE	24.75±3.81	24.46±3.40	28.48±0.44	33.72±0.60	16.77±0.40	16.24±0.48	13.16±1.37	16.72±0.37
	57.34	53.00	64.99	69.43	57.87	62.89	60.52	55.36
	82.09±4.12	77.46±0.92	93.48±2.86	103.16±0.6	74.65±0.26	79.13±0.21	73.68±2.04	72.08±1.73
EM	10.62±0.70	10.06±0.54	12.08±0.20	12.85±0.27	12.89±0.88	12.12±1.56	13.99±0.17	15.28±0.32
	82.14	75.95	69.16	56.01	70.13	57.54	67.05	72.21
	92.77±0.44	86.01±0.20	81.24±0.77	68.86±2.07	83.03±2.69	69.67±0.22	81.05±0.47	87.50±0.49
AP	8.37±0.07	8.61±0.17	10.18±1.48	9.02±0.07	8.57±0.25	5.80±1.28	7.96±0.00	12.42±1.16
	74.73	89.23	87.67	90.52	104.63	93.98	90.51	80.79
	83.10±0.71	97.85±0.92	97.85±0.92	99.55±0.23	113.21±0.1	99.78±0.36	98.48±0.20	93.21±0.07
RW	12.74±0.03	14.19±0.30	15.34±0.30	12.79±0.04	14.00±0.76	12.48±0.38	8.07±1.66	13.30±0.42
	66.46	92.82	95.39	95.57	91.41	76.01	94.25	98.04
	79.18±1.06	107.02±0.5	110.73±1.9	108.37±0.4	105.42±1.5	88.50±3.15	102.32±0.3	111.34±1.1
GW	5.19±0.74	5.05±0.25	8.86±0.33	5.87±0.04	8.13±0.08	7.84±0.10	7.78±0.03	9.91±0.37
	64.76	54.02	36.29	67.31	50.58	36.74	54.19	
	69.96±0.08	59.08±0.06	45.15±0.09	73.18±1.27	58.71±1.25	44.58±0.00	61.98±0.87	
IG	13.07±0.36	11.85±0.23	12.76±0.16			13.93±0.34	11.14±0.83	15.95±0.20
	87.75	83.29	93.19			80.53	94.49	78.12
	100.82±0.5	95.15±0.11	105.95±0.2			94.47±0.17	105.63±0.5	94.07±0.08
JC	20.47±0.48	20.76±0.05	19.39±0.03	21.01±0.48	19.31±0.22	17.22±0.12	17.16±0.17	21.66±0.37
	93.30	95.20	87.69	78.99	93.09	94.77	99.69	127.72
	113.77±1.9	115.97±1.3	107.09±0.2	100.00±2.4	112.40±0.4	112.00±0.2	116.86±0.5	149.38±0.4
MS	13.08±0.11			11.87±0.69	10.21±0.20		12.09±0.31	11.78±0.07
	81.14			84.39	78.22		92.02	83.29
	94.22±0.39			96.26±1.32	88.43±1.75		104.11±0.2	95.07±0.21

Key:

Neopterin

7,8-Dihydroneopterin

Total Pterin

Table IV Continued. Neopterin, 7,8-Dihydroneopterin and Total Pterin Concentrations in STEMI Patients.

	FA	AO pre	CS pre	CA pre	CA post	CS post	AO post	24 hr
CD	16.43±0.06	14.88±0.59	14.47±0.12	14.51±0.04	14.50±0.39	14.51±0.16	14.09±0.00	15.27±0.38
	101.28	101.26	107.93	95.09	108.73	99.05	103.67	121.95
	117.71±0.7	116.15±0.1	122.41±0.6	109.61±2.4	123.23±0.1	113.56±0.9	117.76±1.2	137.23±0.7
JE	7.01±0.19	7.77±0.64	6.21±0.20	5.35±0.09	5.78±0.07	5.85±0.12	5.71±0.12	6.68±0.24
	19.27	15.54	18.86	16.52	16.29	16.33	17.56	15.36
	26.29±0.69	23.31±0.51	25.07±0.25	21.88±0.01	22.08±0.79	22.18±0.29	23.28±0.21	22.04±0.48
LH	6.95±0.29	7.10±0.10	7.53±0.06	8.21±0.20	8.06±0.08	7.09±0.00	7.26±0.06	6.40±0.25
	16.46	17.99	16.22	15.23	14.60	17.73	16.21	11.79
	23.42±0.12	25.09±0.34	23.75±0.01	23.44±7.61	22.66±0.77	24.82±0.14	23.48±0.51	18.19±0.13
TW	3.68±0.48	4.57±0.09	7.03±0.16	4.07±0.29	4.87±0.23	4.68±0.36	4.89±0.48	6.31±0.00
	14.56	14.59	2.79	15.53	11.05	12.21	6.13	15.46
	18.25±0.12	19.17±0.33	9.82±0.11	19.60±0.29	15.92±0.06	16.89±0.38	11.02±0.27	21.77±0.04
RT	7.58±0.04	8.31±0.13		9.00±0.10	7.73±0.80		7.60±0.05	
	18.22	31.57		23.47	29.80		28.37	
	25.81±0.10	39.89±1.85		32.47±0.02	37.53±1.02		35.98±0.27	
CS	19.71±0.55	20.16±1.62	19.20±0.58	19.10±1.31	17.53±1.44	19.34±0.12	17.65±0.73	15.75±0.08
	37.27	50.73	40.66	39.54	44.20	50.79	15.12	50.23
	56.99±0.19	70.89±0.47	59.87±6.80	58.64±8.74	61.74±0.20	70.13±0.00	32.78±1.71	65.98±0.23
RS2				9.95±1.06				7.86±0.40
				42.26				27.92
				52.21±1.13				35.78±0.21
MH	5.98±0.06	6.54±0.14	6.67±0.08	5.08±0.84				5.53±0.30
	26.79	17.74	24.79	22.34				25.65
	32.78±1.71	24.28±0.36	31.47±0.06	27.42±0.65				31.18±0.54
WE	36.42±0.10			32.78±0.68	36.42±0.45			53.70±3.64
	55.05			83.87	86.89			82.38
	91.47±0.03			116.66±0.1	123.32±2.7			136.09±0.2
MF	10.69±1.65		13.18±0.07	10.59±1.37	11.27±0.24		10.47±0.09	13.79±0.22
	26.60		21.42	26.50	23.52		22.69	25.75
	37.29±0.36		34.61±0.25	37.10±0.07	34.80±1.44		33.17±1.05	39.54±0.05
DG	16.57±0.21	9.08±0.38	10.21±0.60	9.83±0.88	8.56±0.42	8.81±0.99	6.92±0.21	7.50±0.07
	23.59	18.36	17.23	4.60	6.99	11.92	22.45	21.62
	40.17±1.54	27.45±6.77	27.44±0.92	14.44±1.45	15.55±3.16	20.73±6.96	29.37±1.54	29.13±0.76
PB	5.79±1.57	7.17±0.14	7.30±0.47	6.44±0.45		6.88±0.28	6.67±0.06	
	19.14	21.72	13.08	15.24		20.09	15.07	
	24.94±0.62	28.90±2.67	20.38±0.31	21.68±0.72		26.97±0.32	21.75±2.79	
AS	6.96±0.28	6.29±0.37	6.19±0.15	6.06±0.19	6.75±0.12	5.86±0.05	6.00±0.18	7.11±0.00
	14.53	17.14	18.08	17.65	24.71	19.51	15.42	7.00
	21.50±2.71	23.44±0.49	24.28±4.52	23.71±4.15	31.47±1.01	25.37±0.08	21.43±0.76	14.11±1.29

Key:

Neopterin

7,8-Dihydroneopterin

Total Pterin

Table V. Neopterin, 7,8-Dihydroneopterin and Total Pterin Concentrations in Stable Chronic Angina Patients.

Concentrations neopterin and total pterin are in nM ± SEM of duplicates. Concentrations of 7,8-dihydroneopterin are in nM, obtained by subtracting the neopterin concentration from the total pterin concentration. FA – femoral artery, AO pre – aorta pre stent, CS pre – coronary sinus pre stent, CA pre – coronary artery pre stent, CA post – coronary artery post stent, CS post – coronary sinus post stent, AO post – aorta post stent, 24 hr – 24 hours after the operation.

	FA	AO pre	CS pre	CA pre	CA post	CS post	AO post	24 hr
	16.05±0.12	18.79±0.17	17.41±0.14	16.87±0.40	19.94±0.74	17.46±0.08	17.12±0.15	
SM	70.23	46.76	47.18	71.30	63.50	73.60	65.09	
	86.29±0.78	65.55±0.04	64.60±0.58	88.17±0.03	83.45±0.75	91.06±0.64	82.22±2.38	
	19.22±0.22	19.94±0.51	17.61±0.13	18.23±0.08	18.07±0.17	18.90±0.10	19.14±0.72	
AW	67.07	58.73	62.12	68.60	65.78	63.21	79.10	
	86.29±0.77	78.68±0.68	79.74±5.93	86.84±2.19	83.86±2.56	82.11±0.40	98.24±2.04	
	51.31±1.64	47.35±2.50	45.47±1.87	38.16±6.63	40.23±0.21	50.00±1.45	38.27±0.57	45.70±0.42
PM	79.64	57.65	97.75	49.31	52.41	47.23	50.71	71.17
	130.9±21.2	105.00±2.0	143.22±9.7	87.48±4.52	92.64±0.34	97.24±0.42	88.99±6.33	116.87±0.1
	11.80±0.19	11.34±0.08	11.39±0.27	10.42±0.05		9.82±0.05	9.73±0.07	10.13±0.22
BS	51.38	47.16	47.27	57.67		48.78	55.36	63.15
	63.18±4.99	58.51±0.23	58.67±6.91	68.10±7.60		58.61±2.83	65.09±6.32	73.28±8.92
	6.33±1.51	2.11±0.31	3.18±0.20	3.08±0.65	5.54±0.17	2.78±0.01	7.12±1.41	8.37±2.08
AMZ	31.28	30.51	33.28	59.36	48.07	28.55	50.93	31.37
	37.61±1.58	32.62±1.99	36.46±0.65	62.45±2.81	53.62±4.56	31.34±0.04	58.06±2.08	39.74±1.81
	19.35±0.34	18.85±1.22	17.36±1.21	17.44±0.37	20.74±0.66	18.13±0.38	17.91±0.03	17.37±0.07
MJ	44.29	50.59	45.03	48.27	34.43	36.52	46.57	55.98
	63.64±0.20	69.44±2.09	62.39±0.20	65.71±0.01	55.17±0.72	54.66±0.02	64.49±0.02	73.36±0.84
PN	10.34±1.95	10.58±1.47	11.41±0.14	8.24±2.24	11.63±0.24	9.40±2.86	10.46±0.35	14.21±0.10
	14.02±0.70	14.10±0.07		14.73±0.57	13.88±0.31		15.18±0.08	15.39±0.13
CT	101.73	87.17		84.86	73.99		57.26	65.11
	115.76±1.4	101.28±1.4		99.60±0.80	87.87±0.27		72.45±0.08	80.50±0.34
	14.00±1.02			11.50±0.14	12.07±0.00		13.88±0.00	12.05±0.44
RG	95.70			69.47	104.02		81.17	83.68
	109.71±0.7			80.97±0.73	116.10±0.4		95.05±2.26	95.73±0.04
	9.81±0.28	9.00±0.04	9.31±0.09					
KD	87.70	78.22	74.90					
	97.52±0.81	87.22±1.03	84.22±0.66					
	13.76±0.32	15.42±0.54		15.26±0.45	14.21±0.40		14.15±0.05	
BF	23.53	30.47		32.44	31.78		31.34	
	37.30±0.28	45.89±0.02		47.70±0.09	45.99±0.41		45.49±0.46	
	7.16±0.13	7.36±0.00	6.95±0.05			4.77±1.15	4.48±0.77	6.27±0.06
DD	23.21	30.42	15.79			20.28	18.69	31.51
	30.38±0.79	37.78±0.19	22.75±0.32			25.05±0.32	23.18±1.03	37.78±0.19

Key:

Neopterin

7,8-Dihydroneopterin

Total Pterin

Table VI. Neopterin, 7,8-Dihydroneopterin and Total Pterin Concentrations in the Control Group of Healthy Volunteers.

Concentrations neopterin and total pterin are in nM ± SEM of duplicates. Concentrations of 7,8-dihydroneopterin are in nM, obtained by subtracting the neopterin concentration from the total pterin concentration. All samples were taken from the forearm vein.

	Neopterin	7,8-Dihydroneopterin	Total Pterin
RP	3.28±0.40	40.67	43.96±0.60
MH	16.13±0.23	27.44	43.57±0.02
BD	44.09±0.01	22.38	66.48±0.62
UF	4.89±0.29	16.98	21.87±0.64
LX	6.88±0.01	18.46	25.35±0.30
H 1	5.02±0.03	17.18	22.20±0.47
H 2	3.09±0.17	21.14	24.24±0.85
H 3	1.24±0.04	18.10	19.35±0.41
H 4	1.68±0.31	15.07	16.75±0.30
H 5	5.76±0.01	16.90	22.67±0.69
H 6	6.10±0.16	18.06	24.16±0.51
H 7	5.03±0.01	10.46	15.49±0.46
H 8	4.42±0.16	5.81	10.24±0.04
H 9	4.62±1.06	15.19	19.81±7.23
H 10	5.91±0.67	11.62	17.53±0.20
H 11	2.61±0.15	20.85	23.47±0.43
H 12	4.97±0.16	7.30	12.28±0.34
H 13	5.37±0.71	17.97	23.35±0.73
H 14	3.99±0.44	19.81	23.81±0.44
H 15	4.42±0.18	15.11	19.53±0.08
H 16	6.52±0.02	28.21	34.73±0.80
H 17	4.10±0.11	6.45	10.55±1.04